1989

Selected toxicological studies of the mycotoxin cyclopiazonic acid in turkeys

Charles David Miller
Iowa State University

Order Number 8920169

Selected toxicological studies of the mycotoxin cyclopiazonic acid in turkeys

Miller, Charles David, Ph.D.

Iowa State University, 1989

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Selected toxicological studies of the mycotoxin cyclopiazonic acid in turkeys

by

Charles David Miller

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Veterinary Pathology
Interdepartmental Major: Toxicology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Interdepartmental Program

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Members of the Committee:

Signature was redacted for privacy.

Iowa State University
Ames, Iowa

1989

TABLE OF CONTENTS

GENERAL INTRODUCTION

Cyclopiazonic acid is a toxic secondary metabolite produced by several different species of the fungal genera, Penicillium and Aspergillus. The toxicity of CPA has been demonstrated in several animal species including rats,[1-10] mice,[11-14] guinea pigs,[15-17] chickens,[18-23] dogs,[24] rabbits,[25] pigs,[26] and monkeys.[27] The known clinical signs include decreased weight gain, diarrhea, dehydration, depression, opisthotonos, convulsions, hyperesthesia, hypokinesis, vomition, and death. Lesions resulting from CPA administration have occurred in the gastrointestinal tract, liver, kidneys, spleen, pancreas, salivary glands, heart, and skeletal muscle. The extent of CPA contamination of food and feed has not been fully evaluated; however, corn, peanuts, and cheese have been shown to be naturally contaminated with CPA.[28-31]

The role of CPA in animal and human disease has not been established. However, because of the number and variety of fungal species that produce CPA, and because many of these fungi are present in crops of worldwide importance such as peanuts and corn, this mycotoxin merits toxicological investigation.

This study was undertaken because no toxicity studies have been conducted with turkeys. Both in vitro and in vivo evaluations were conducted to evaluate cardiac effects of CPA. Physiological parameters, histological and ultrastructural evaluations were conducted. This dissertation consists of a series of manuscripts to be submitted to the

American Journal of Veterinary Research. A general summary and discussion follows the last manuscript. Literature cited in the dissertation, introduction, literature review, general summary, and discussion appear at the end of the dissertation.

LITERATURE REVIEW

Historical and general chemical perspective Scientific study of the mycotoxin, cyclopiazonic acid (CPA) has spanned 25 years. Cyclopiazonic acid was initially identified in 1968 during a metabolite screening study of Penicillium cultures.[32] Retrospectively, CPA was incriminated in turkey deaths observed in the "turkey X" disease outbreak of 1960.[33] Initial experimental work with CPA began in 1971 when Purchase[1] determined the oral (po) and intraperitoneal (ip) LD_{50} for rats.

Cyclopiazonic acid has been characterized as an indole tetramic acid that acts as a mono-basic acid. Cyclopiazonic acid is derived from one molecule of tryptophan, one molecule of mevalonic acid, and two molecules of acetic acid. Its molecular weight is 336.15 and molecular formula is $C_{20}\ H_{20}\ N_2\ O_3$.[32,34,35] From methanol extracts crystalline CPA can be isolated and it has a melting point of 245 - 246°C.[36,37]

Cyclopiazonic acid is produced by several species in the genera Penicillium and Aspergillus. Natural contamination with CPA has been determined in corn,[28] peanuts,[29] and cheese.[30,31,38] There is presumptive evidence that CPA could be present in a number of other commodities because they contained CPA-producing fungi. These commodities included: navy beans, pinto beans, corn meal, macaroni, pecans, walnuts, ham, sausage, and frankfurters.[30,39,40]

Presently several methods are utilized for analytical detection of CPA and include thin-layer chromatography,[18,41] nuclear magnetic resonance analysis,[42] reversed phase high-performance liquid chromatography,[29] and normal phase high-performance liquid chromatography.[43] A simple colorimetric method for screening CPA in agricultural commodities was recently reported.[44]

Cyclopiazonic acid is readily absorbed from the gut, with rapid distribution and excretion in rats. Following administration of radiolabeled CPA, a half life of 33 $\pm$ 12 hours was established for intraperitoneal and 43 $\pm$ 12 hours for intragastric exposures. CPA was found in various tissues but no bioaccumulation occurred in tissues. The highest levels of CPA were found in blood, skeletal muscle and liver, respectively. Biliary excretion had a major role in CPA excretion in feces.[7]

The mutagenic activity of CPA has been studied in Salmonella typhimurium and the results are contradictory; Wehner et al.[45] reported CPA had no mutagenic response. However, Sorenson et al.[46] reported a positive response. An older report[47] suggested that CPA may be carcinogenic and responsible for liver carcinomas in Bantu tribes of Africa. Teratologic studies have been conducted with mice, rats, and chickens. No teratogenic effects were observed in rats and mice; however, teratogenic effects were observed in 2-day old chicken embryos in which the caudal end of trunk was shortened and microphthalmia occurred.[19]

Toxicity The acute oral LD_{50} in male rats given CPA is 36 mg/kg and 63 mg/kg for females. The intraperitoneal (ip) LD_{50} for male

rats is 2.3 mg/kg.[1] The ip LD_{50} in male mice was found to be 13 mg/kg.[12] The acute oral LD_{50} in day-old cockerels was found to be 21.71 mg/kg.[19] Another study determined a 72-hour LD_{50} of 12.0 mg/kg for male broiler chicks and 12.1 mg/kg for females.[23]

The response to CPA is both species and dose related. Synopsis of several studies with CPA that have produced variable results depending on the species of experimental animal, sex, route, dose, carrier, and duration of administration are shown in Table 1.

Pathophysiology Animals exposed to CPA usually show: (i) clinical pathological changes; (ii) gross pathological changes; and (iii) microscopic alterations.

(i) Clinical pathological changes. Changes in some clinical pathological values following CPA exposure have been reported in chickens,[20,23] dogs,[24] guinea pigs,[17] and rats.[6] Chicks gavaged with 2 or 4 mg/kg of CPA in corn oil had increased plasma creatinine kinase activity that correlated with skeletal muscle degeneration.[20] In a similar study, a dose-related decrease in hematocrit, plasma potassium and protein was noted in chicks dosed with CPA at 1, 2 or 4 mg/kg for 23 days.[23] In guinea pigs exposed to CPA at doses ranging from 0.00625 to 1.95 mg/day for 30 days, the most remarkable changes in any measured parameter were in the serum enzyme concentrations. The aspartate amino-transferase (AST), alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) concentrations were increased, but only in the animals given 1.6 and 1.95 mg CPA/day. Total serum bilirubin concentration increases were slightly elevated with increasing doses of CPA. Total

Table 1. Experimental Toxicity of CPA for Pigs, Poultry, and Laboratory Animals

Species	Route/Dose	Duration	Clinical Signs	Reference
Piglets	po/ 10 mg/kg	14 days	Anorexia, inactivity, rough hair coat, weakness	Lomax et al. 1984
Piglets	po/ 1 mg/kg	14 days	Inactivity, rough hair coat	Lomax et al. 1984
Chicks	po/ 4 mg/kg	23 days	Deaths	Cullen et al. 1988
Chicks	po/100 ppm	49 days	Deaths, decreased weight gain	Dorner et al. 1983
Chickens	po/ 10 mg/kg po/ 5 mg/kg	4 days	Decreased weight gain	Norred et al. 1988
Chicks	po/ 4 mg/kg	23 days	Decreased weight gain, feed and water intake	Wilson et al. 1986
Dogs	po/ 1 mg/kg po/ 0.5 mg/kg	90 days	Deaths, anorexia, vomiting, diarrhea, weight loss, pyrexia, depression	Neuhring et al. 1985
Monkeys	po/ 20 mg/kg	112 days	Sporadic emesis, no weight gain	Jaskiewicz et al. 1988
Rabbits	ip/ 10 mg/kg	$\leq$1 day	Tachycardia, dyspnea, death	Nishie et al. 1985

Table 1 (Continued)

Species	Route/Dose	Duration	Clinical Signs	Reference
Mice	ip/ 11 & 14 mg/kg	6 days	Complete feed and water refusal, tremor, prostration, ptosis, death	Nishie et al. 1985
Mice	ip/ 10 mg/kg	1 day	Catalepsy, weight loss	Nishie et al. 1985
Rats	ip/ 8 mg/kg	<1 day	Ataxia, death,	Purchase 1971
	po/ 30 to 83 mg/kg	1.5 - 2 days	prostration, death	Purchase 1971
Rats	po/ 8 mg/kg	4 days	Diarrhea, nasal discharge, rough hair coat, ataxia, lethargic, deaths	Morrissey et al. 1985
Rats	ip/ 5 mg/kg	28 days	Diarrhea, dehydration	Hill et al. 1986
Guinea pigs	po/1.6 & 1.95 mg/day	30 days	Death, anorexia, rough hair coat, ataxia, pain upon movement, diarrhea	Richard et al. 1986

protein and albumin concentrations were not changed, except a slight increase was noted in the animals given 1.6 and 1.95 mg/day.[17] In rats exposed to 8.0 mg/kg for 4 days, serum AST and ALT were elevated, cytochrome P-450 was decreased and glutathione S-transferase was unchanged. No serum chemical changes were noted in the rats given 0.2, 2.0, and 4.0 mg/kg/day.[6] Hill et al.[8] reported that in rats given 0.1, 1.0, and 5.0 mg/kg for 28 days, total protein, albumin, PCV and hemoglobin values were not significantly altered. The rats given 1.0 and 5.0 mg/kg had higher mean neutrophil counts and lower mean eosinophil and lymphocyte counts than did controls. Cyclopiazonic acid did not affect the degree of cellularity, myeloid to erythroid ratio and presence of all stages of maturation in bone marrow smears. Dose-related clinical pathologic changes in dogs dosed at 0.05, 0.5 and 1.0 mg/kg, consisted of leukocytosis, neutrophilia, lymphopenia, monocytosis and increased serum alkaline phosphatase activity.[24] Pigs treated with CPA at doses ranging from 0.01 to 10 mg/kg, showed no changes in PCV, plasma protein hemoglobin, serum calcium, phosphorus and magnesium, total protein, albumin, blood urea nitrogen, serum alkaline phosphatase, AST, and gamma glutamyl transpeptidase.

(ii) Gross pathological changes. Relatively few gross lesions have been observed in animals following exposure to CPA. Splenic changes including atrophy, necrosis, hemorrhage and mucosal erosions in the gastrointestinal tract have been reported in the rat.[1,2,6] In 500 g guinea pigs given 1.6 and 1.95 mg/d, superficial necrosis of the gastric mucosa was the significant lesion reported.[17] Chickens fed 50 or 100

ppm had lesions in the gastrointestinal tract. Thickened mucosa and dilated lumens were noted in the proventriculi of birds fed 50 ppm. Birds from the 100 ppm group had proventricular mucosal erosions and hyperemia. Splenic necrosis was reported in both 50 and 100 mg/kg birds.[18] Similar gross lesions including; hemorrhage, necrosis and hyperplasia of proventricular mucosa were reported in chickens gavaged with CPA.[20] Dogs appear to be very susceptible to CPA. Oral doses of 1.0 mg/kg caused death at 48 h. Gross lesions were frequently observed in kidneys and the gastrointestinal tract. Kidney lesions included raised circular infarcts. Ulceration of the soft palate, esophagus and gastrointestinal tract was seen. Other lesions observed were nuclear enlargement in the bladder, epithelium and adrenal glands, and necrosis in the skin and epididymis.[24] Lomax et al.[26] reported lesions in 1 and 10 mg/kg treated piglets. Lesions were most prominent in the kidneys and gastrointestinal tract. Gross lesions of gastric hemorrhage, ulceration and mucosal hyperemia were observed in both the large and small intestines. No gross lesions were reported in monkeys given up to 60 mg/kg.[27]

(iii) Microscopic alterations. Target organs vary in different laboratory animal species treated with CPA. Rats given oral doses from 2.5 to 8.0 mg/kg had liver changes that were dose related progressing from single cell necrosis to multiple foci of necrosis. Changes in bile ducts included karyomegaly and swollen cells that occluded the duct. Bile duct epithelial necrosis was reported at 4.5 mg/kg.

Pancreatic changes included necrosis of acinar cells, vacuolar degeneration and pyknosis of islet cells.[1,2,4] In a low dose (0.1 to 5.0 mg/kg) chronic study involving rats, only hepatic and renal changes were noted. Changes included vacuolated and granular hepatocytes, dilated renal tubules with pyknotic nuclei and a few scattered casts.[8] In addition to renal and hepatic lesions, salivary gland changes consisting of swollen serous and ductular epithelial cells, with clear nucleoplasm and prominent nucleoli were reported in rats orally dosed for 21 days.[3] In two studies of chickens orally exposed to CPA, gastrointestinal changes were the most remarkable. Dorner et al.[18] reported mucosal epithelial necrosis and inflammation in the crop, proventriculus, and ventriculus. Hepatocellular vacuolation and skeletal muscle degeneration also were reported following CPA gavage.[20] Guinea pigs had lesions similar to those seen in rats, including gastric mucosal necrosis and hepatocellular vacuolation at CPA doses of 1.6 and 1.95 mg/day.[17] Piglets given oral CPA at 10 mg/kg/d had microscopic lesions in gastric and intestinal mucosa, liver and kidneys. Gastric mucosal inflammation and necrosis, villous blunting, and mucosal hyperemia and hemmorhage in both large and small intestinal tracts was noted. Only mild focal gastric mucosal necrosis was seen in piglets dosed at 1.0 mg/kg/d.[26]

Dogs are the most sensitive species that have been tested for toxicity to CPA. In feeding studies conducted with dogs, CPA levels of 0.1, 1.0 and 2.0 mg/kg were administered. The major dose-related microscopic lesions seen in different tissues were categorized as: vascular damage, ulceration accompanied by necrosis, and necrosis with

karomegaly. Ulcerative and necrotic lesions were associated with vascular damage in all systems, except for the lymphoid organs. Vascular injury was random and segmental with changes that included: swollen vacuolated endothelial cells, acidophilic granular material between the endothelium and internal elastic membrane, loss of endothelial cells, occurrence of fibrin in the tunica media, vacuolation of myocytes of the tunica media and infiltrates of esosinophils and neutrophils. Severely affected vessels had foci of necrosis, hemorrhage and neutrophils in the intima and media. Gastrointestinal erosions and ulcers had surrounding mucosal lamina propria that was hyperemic, edematous and infiltrated with neutrophils. Necrosis was observed in kidneys, livers and lymphoid tissues of the 1.0 mg/kg dogs. Karyomegaly was observed in the liver, kidney, gastrointestinal tract, urinary bladder epithelium and adrenal glands.[24] In monkeys exposed to levels of CPA up to 60 mg/kg/d for 16 weeks, microscopic lesions were mild and irregular. Mild karomegaly of biliary and pancreatic duct epithelial cells, and renal medullary tubules were noted; minute tubular atrophy and protein casts were present.[27]

Liver, cardiac, and skeletal muscle ultrastructural changes have been reported following exposure to CPA.[5,15,27] Oral dosages of 2.0 and 4.0 mg/kg/day resulted in swollen mitochondria and vesiculation of the rough endoplasmic reticulum in rat livers.[5] Ultrastructural changes were also reported in guinea pigs receiving total dosages of 4, 8, 12, and 16 mg/kg. Limited mitochondrial swelling was found in guinea pigs given the lower doses. Animals that received 12 and 16 mg/kg

showed a dose response in severity and degree of dilated sarcoplasmic reticulum, swollen mitochondria and segmental necrosis in gastrocnemius and vastus lateralis musculature.[15] Unusual irregular thickening and cytoplasmic vacuoles or abnormally large fenestrations were the most pronounced ultrastructural changes found in cardiac capillary endothelium and kidneys of monkeys exposed to CPA. Cytoplasmic processes of long, irregular foldings were noted. Some capillary endothelium appeared to be separated from the basal membrane with accumulation of homogenous substance in the subendothelial space. Myocytes had focal lysis of myofibrillae, mitochondrial accumulation and lysosome formations. Hepatocytes had dilated rough endoplasmic reticulums.[27]

Cardiac pathophysiology Several studies reported that CPA produced lesions in or altered function of the heart. Purchase[1] first reported cardiac lesions of hyaline or coagulative degeneration of rat myocardium following CPA exposure to oral dosages ranging from 30 to 82.6 mg/kg. Findings in a distribution study indicated that heart, along with lungs, kidneys, liver, and skeletal muscle were the tissues with the greatest amounts of labelled CPA.[7] Altered cardiac function reported in rabbits exposed to 5.7 or 10 mg/kg of CPA was characterized by tachycardia followed by bradycardia and terminal ventricular fibrillation.[11] Negative inotropic effects in isolated frog ventricles were observed 30 seconds after exposure to 20 ug/ml of CPA.[48] In chickens, fed 50 and 100 ppm dietary CPA on an *ad libitum* basis, there was subacute to chronic inflammation of the epicardium and chronic myocardial inflammation noted at both dosing levels.[18] Decreased heart muscle

mass was reported in broiler chicks orally exposed to CPA at 1, 2, 4, 8, and 16 mg/kg.[23] Other myocardial pathological effects have included cellular degenerative changes of enlarged pleomorphic nuclei, with margination of chromatin following weekly CPA dosages of 12 or 21 mg/kg in rats.[3] Myocardial lesions were present in two monkeys treated with CPA; doses up to 60 mg/kg caused lesions of focal degenerative cellular changes, focal atrophy and cellular disarray were reported.[27] In contrast, no heart lesions were noted in CPA studies where oral dosages were 4 mg/kg in rats;[10] 1, 2, or 4 mg/kg in broiler chicks;[20] 0.05, 0.25, 0.5, or 1.0 mg/kg in dogs;[24] and 0.01, 0.1, 1.0 or 10 mg/kg in piglets.[26]

Cyclopiazonic acid in vitro assays In vitro experiments utilizing CPA have included tissue preparations of esophagus, jejunum, ileum, trachea, aorta, vas deferens, uterus, ventricles, cell cultures of rat hepatocytes and pig kidney epithelial cells and sarcoplasmic reticulum vesicles from rat skeletal muscle.[48-52] Cyclopiazonic acid enhances peristaltic movement of the jejunum, ileum, and estrous uterus and produced broncho-constriction in vitro. The inotropic effects of dopamine, epinephrine and serotonin in vas deferens were not blocked by CPA. These findings indicated that the reported toxic effects of CPA (hypothermia, catalepsy, hypokinesia, and tremor) probably were not due to neurotransmitter-receptor blockage.[48] Altered membrane permeability or alteration of ion pumps were hypothesized to be the mechanism of action when CPA stimulated accumulation of a transmembrane potential

probe, tetraphenylphosphonium bromide (TPP), in cultured pig kidney renal epithelial cells.[49]

Subsequent studies utilized inhibitors of TPP accumulation and fractionation of renal epithelial cell to examine CPA membrane effects. Cells accumulated TTP in plasma membrane and mitochondrial fractions when exposed to CPA. Control cells accumulated TPP in the cytosol. These findings suggested that CPA may: (1) combine with TPP within membranes, (2) act as a TPP carrier, or (3) create a negative electrostatic potential within membranes.[49,51] Other studies have also shown that CPA alters calcium flux in renal epithelial and skeletal muscle cells[51] and is a potent inhibitor of calcium ion transport and calcium ATP-ase in rat skeletal muscle sarcoplasmic reticulum.[52]

In other studies utilizing CPA alone or in combination with aflatoxin B_1 there has been negative or equivocal results on immunological function.[17,53]

SECTION I. *IN VITRO* EVALUATION OF ISOLATED TURKEY CARDIAC MUSCLE FUNCTION

In Vitro Evaluation of Isolated Turkey Cardiac Muscle Function

C. D. Miller[1], DVM, MS, F. B. Hembrough[2], DVM, PhD, J. L. Richard[1], PhD, G. D. Osweiler[3], DVM, PhD

[1]National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, P. O. Box 70, Ames, IA 50010.

[2]Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

[3]Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

SUMMARY

Turkey papillary muscles can function for up to six hours in an isolated chamber containing oxygenated, pH-regulated, modified Krebs-Henseleit solutions. Our data indicate that different calcium concentrations affect muscle contraction and relaxation velocities, load values, and latencies. This controlled *in vitro* biological assay system can be used in further studies to evaluate species specific cardiac toxins or drugs.

INTRODUCTION

Papillary muscles from a number of species including: cats,[1-4] dogs,[5-6] pigs,[7] rats,[8] guinea pigs,[9] rabbits,[10-11] ferrets,[12] and hamsters[13] have been utilized to study cardiac mechanics. Additionally, these muscle preparations have provided an in vitro evaluation of the physiological and pharmacological effects of toxins, drugs, and hormones[14-16] on cardiac function. There has been comparatively little work done with isolated cardiac muscle preparations from the avian species. A turkey cardiac muscle in vitro assay system will provide additional avian cardiac mechanics data and a means to study known, suspected, or species-specific cardiotoxins.

The objectives of this study were to determine the experimental conditions and techniques that would optimize in vitro turkey papillary muscle function. We have reported on initial investigations that dealt with muscle harvest techniques, solution modifications and temperature evaluations for optimal muscle function.[17] Specific attention was directed towards defining optimal calcium concentration and appropriate evaluation criteria. This report provides baseline information on in vitro function of turkey papillary muscle preparations.

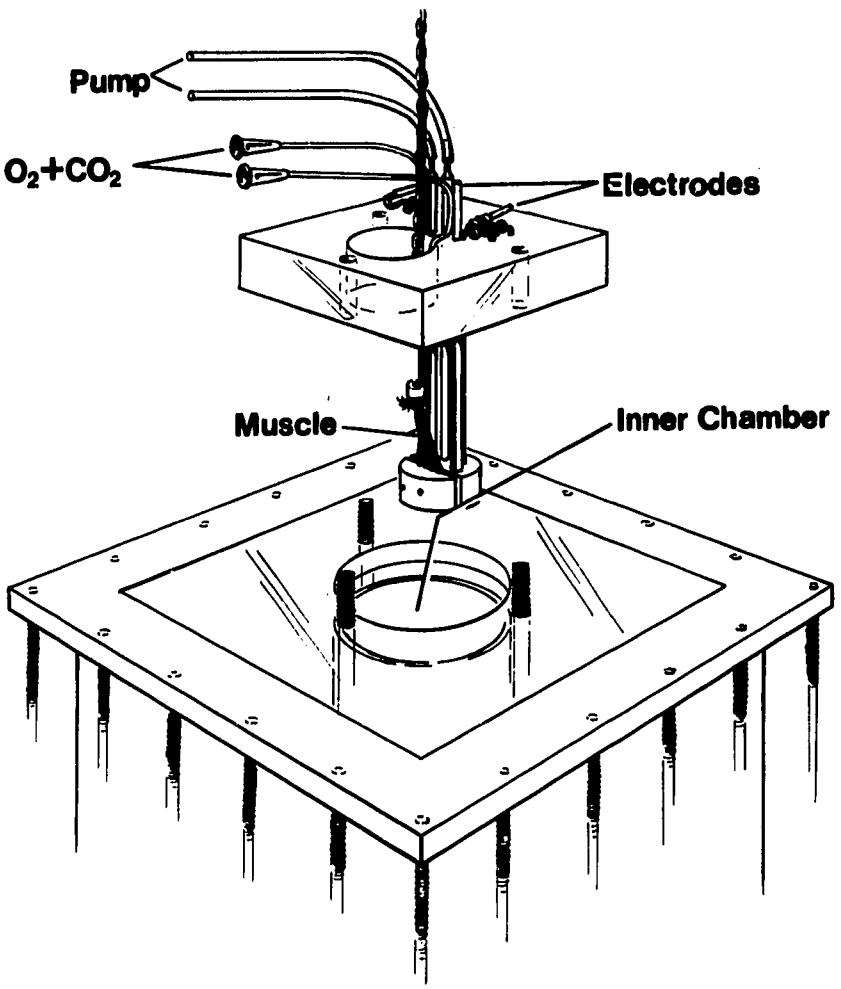
MATERIALS AND METHODS

Turkey papillary muscle preparation Twenty turkey poults (8-14 weeks old, Broad Breasted White) were anesthetized with sodium pentobarbital (65 mg/kg). The heart was removed and left ventricular papillary muscles were rapidly cut to the dimensions of 1 mm X 1 mm X 7-10 mm[17] while the tissue was immersed in an oxygenated,[18] modified Krebs-Henseleit (K-H) solution.

Equipment Papillary muscles were mounted vertically in the inner chamber (10 ml) of a double chambered constant-temperature bath as shown in Figure 1. The non-tendinous end of the muscle was mounted in a lucite clamp. The tendinous end was secured in a stainless steel hook with sliding clamp. This hook was attached to a silver chain whose distal end was connected to an aluminum lever mounted on a Brush transducer. The muscles were suspended in a modified K-H solution with the following basic composition (mM): NaCl (118); KCL (4.7); $MgCl_2$ (1.2); $Na_2H_2PO_4 \cdot H_2O$ (11); $NaHCO_3$ (24.0); $CaCl_2 \cdot 6H_2O$ (1.7); glucose (4.5); pH 7.40. The chamber fluid was varied by changes in calcium concentrations. Arbitrary values of 5.5 mM, 4.32 mM, 3.5 mM, and 1.7 mM calcium were utilized. These values were based on literature values, experimental results primarily from dog papillary muscles, and preliminary turkey papillary muscle experiments.

Circulation of the chamber fluid was accomplished through the use of an individual rate-controlled pump. The K-H solution was pumped from the chamber via a stainless steel tube (20 ga) mounted on the end of the

Figure 1. Schematic diagram of the chamber for turkey papillary muscle bioassay studies


Pump
O_2+CO_2
Electrodes
Muscle
Inner Chamber

inner muscle chamber just below the fluid surface, into the pH monitoring chamber. A roller pump returned the fluid to the muscle chamber via a second stainless steel tube (20 ga). This tube was mounted directly under the muscle. The total system required 10.5 to 11.0 ml of fluid. The fluid was aerated by bubbling 95% O_2 - 5% CO_2 into the K-H solution in the mounting chamber. Fluid pH was continuously measured and temperature was maintained at 31° C.

A 4-channel Beckman polygraph R-611 (No. 611, Beckman Instruments Inc., Electronic Instruments Division, Dept. 205, 3900 River Road, Schiller Park, IL) was used to record time, muscle contraction, and stimulus applied to the muscle.

Contractile studies Muscles were allowed to stabilize for 2-2 1/2 hours while contracting isotonically in K-H solution. Following stabilization, the length of muscle that produced maximal contraction (L max) was determined for each muscle preparation. The procedures for determining L max and 90% L max were the same as previously described.[19]

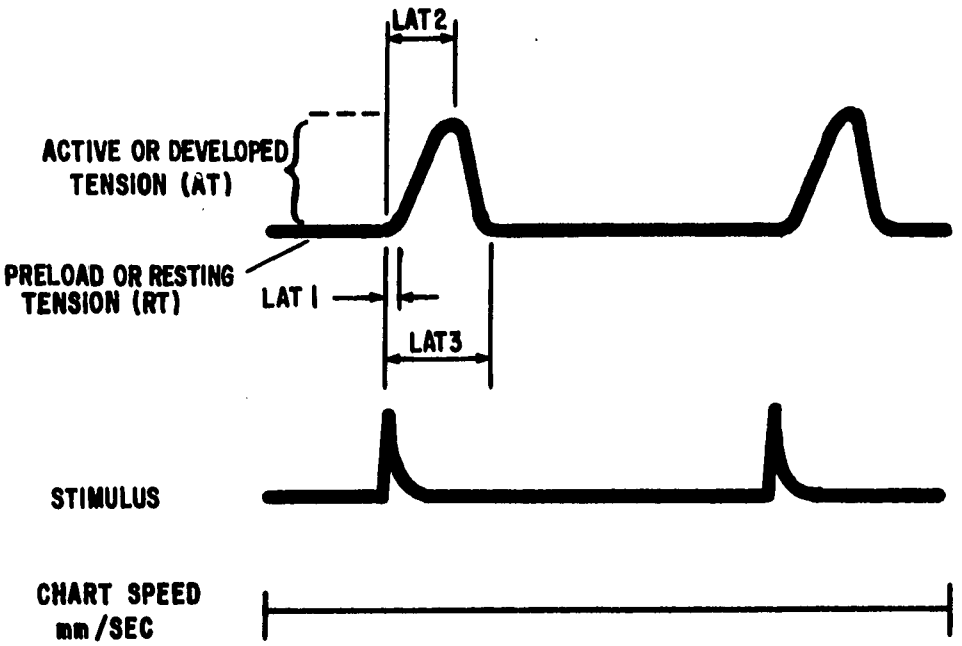
Seven parameters of muscle function were obtained from afterloading turkey papillary muscles. These parameters were: the maximum weight a muscle could lift (P_o); maximum contraction and relaxation velocities (V max); time delay or latency between electrical stimulation and initiation of contraction (Lat 1), peak contraction (Lat 2), or completion of contraction (Lat 3) and distance a weight was lifted (Work). V max was determined by the Hill equation from contraction or relaxation values generated by each afterload curve. Latency values were measured from one weight that corresponded to the preload weight or 90% L max weight.

Latency values were also derived from the contraction curves as shown in Figure 2. Each set of muscle afterload data was normalized by length, cross-sectional area, and P_o for each muscle.

Solution studies The experimental (K-H) solution was prepared on the experimental day. Osmolarity and pH were recorded. At the completion of a 2 to 2 1/2 hour isotonic stabilization period and preload determination, the K-H solution was changed. Solution pH was maintained at 7.4. The muscle was allowed to equilibrate for a 20 minute period in the fresh K-H solution and an afterload curve was generated. Five muscles were tested with each solution before the next solution was utilized. The order of experimental completion was 5.5 mM, 4.32 mM, 3.5mM, and 1.7 mM calcium in K-H solutions.

Statistical analysis Afterload curve contraction and relaxation velocity data were fit to a 3rd degree polynomial equation. Analysis of variance was used to compare the average V max, P_o, Work, and latency values.

Figure 2. Evaluation parameters for latency from turkey papillary muscle bioassay contraction recordings. LAT 1 = time to onset of contraction; LAT 2 = duration of contraction; LAT 3 = total duration of contraction and relaxation


LAT2
ACTIVE OR DEVELOPED TENSION (AT)
PRELOAD OR RESTING TENSION (RT)
LAT I
LAT3
STIMULUS
CHART SPEED mm/SEC

RESULTS

The normal plasma values obtained from our preliminary evaluations of young turkeys (Table 1) were the criteria used in developing the modified K-H solution utilized in this study. The effects of altering calcium concentrations on muscle performance are shown in Table 2. These afterload data indicate that there were significant differences among the means of four parameters (P_o, V max, Work, Lat 3) and no significant differences among the Lat 1 and Lat 2 means. Mean comparisons for each group of the five significant calcium related effects are shown graphically in Figure 3. Comparisons were considered significantly different at the $P \leq .05$ level. P_o comparisons indicated that the 1.7 mM calcium group was significantly lower than the other treatment groups. There were no differences between the 3.5 mM, 4.32 mM, or 5.5 mM calcium means.

We found that both the maximum contraction and relaxation velocities data showed 5.5 mM, 4.32 mM, and 3.5 mM calcium groups were significantly different from each other. There were no differences among 1.7 mM and 3.5 mM calcium groups but graphically there was a decreasing trend.

The 5.5 mM and 1.7 mM treatment groups were significantly different from the two other calcium groups when comparing the muscles's ability to do work. There was no difference among the 4.32 mM and 3.5 mM calcium groups.

The 5.5 mM calcium group was significantly lower than the other groups when measuring Lat 3. There were no significant differences among

Table 1. Mean normal plasma values of total glucose and selected elements from young turkeys (8-14 wk.)

Determination	Normal Value[a,b]
Calcium[c]	12.6 ± (00.6) mg/100 ml
Magnesium	2.3 ± (00.3) mg/100 ml
Glucose	235.0 ± (59.0) mg/100 ml
Sodium	154.0 ± (16.0) meq/l
Potassium	5.2 ± (00.9) meq/l

[a] Numbers in brackets represent standard deviations.

[b] Based on N = 25 birds.

[c] Normal value expressed in mM is 3.09 ± 0.15.

Table 2. Mean contraction and relaxation velocities, P_o, work and latency afterload values in response to different calcium concentrations from turkey poult papillary muscles

	Calcium Concentration (mM)			
	1.70	3.50	4.32	5.50
Contraction				
Maximum Velocity				
(mm/sec/CSA)[a,b,c]	0.40 ± 0.08	0.49 ± 0.14[+]	0.89 ± 0.22[+]	2.03 ± 0.29[+]
Relaxation				
Maximum Velocity				
(mm/sec/CSA)[a,b,c]	0.29 ± 0.05[*]	0.51 ± 0.07[+]	0.92 ± 0.13[+]	2.93 ± 0.31[+]
P_o (mg)[a,c,d]	0.21 ± 0.04[*]	0.34 ± 0.05	0.34 ± 0.09	0.30 ± 0.06
Work (mgs·mm/CSA)[a,c]	60.70 ± 10.60[†]	159.00 ± 21.30	173.00 ± 36.20	394.00 ± 105.20[†]
Latency 1 (mm)[c]	1.58 ± 0.16	1.55 ± 0.29	1.65 ± 0.45	1.55 ± 0.40
Latency 2 (mm)[c]	7.10 ± 0.44	7.23 ± 0.30	6.93 ± 0.47	6.75 ± 0.13
Latency 3 (mm)[c]	11.40 ± 0.69	10.90 ± 0.18	10.80 ± 0.27	9.85 ± 0.47[§]

[a] Normalized for muscle cross sectional area (CSA).

[b] Maximum contraction and relaxation velocities are y intercepts, determined by plotting velocity vs load.

[c] Mean and S.E.M.

[d] P_o is the maximum weight a muscle could lift.

* Value significantly different ($P \leq 0.05$) from 3 other groups.

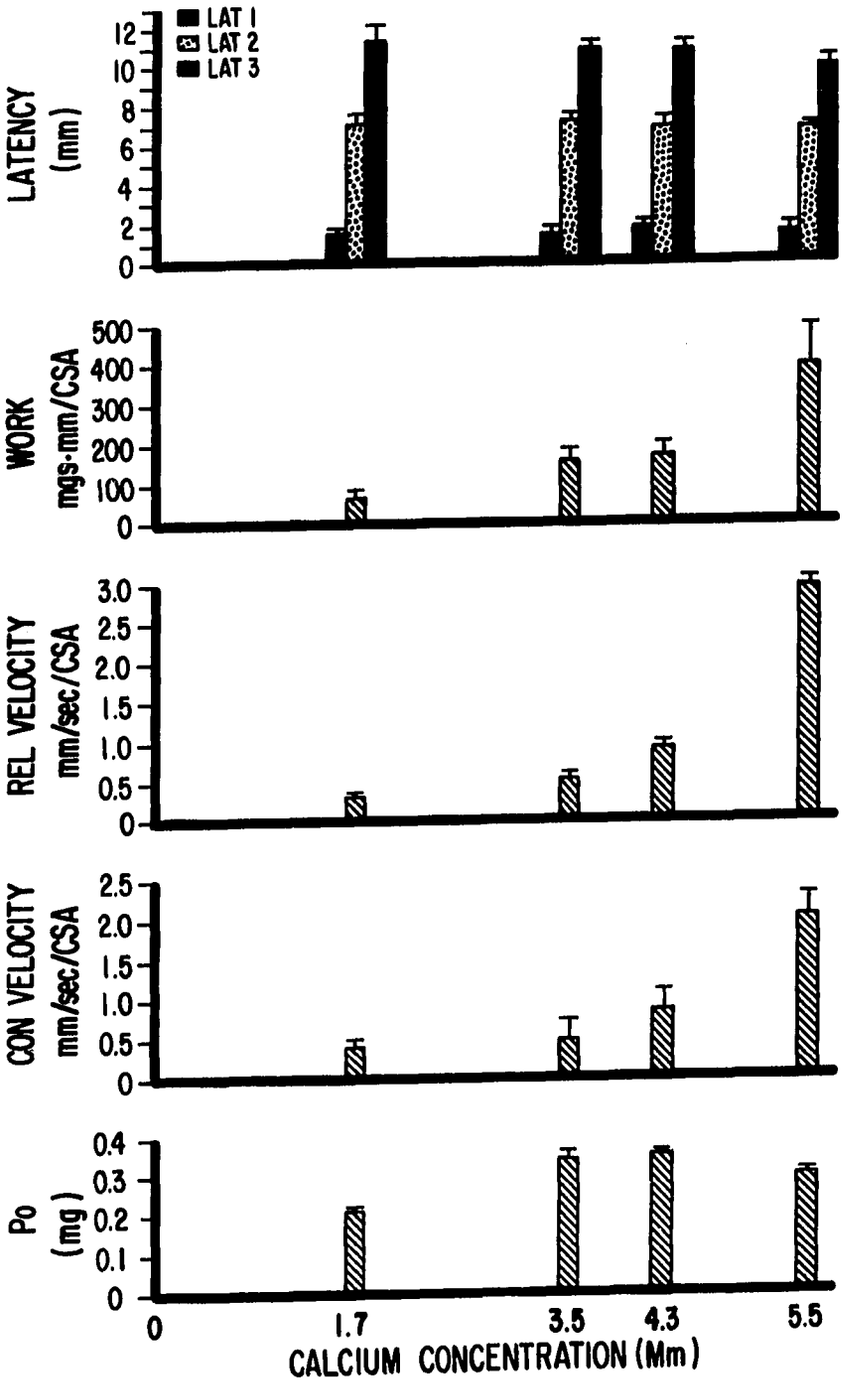
+ Value significantly different ($P \leq 0.0001$) from 1.7 mM group.

† Value signficantly different ($P \leq 0.001$) from 2 other groups.

§ Value significantly different ($P \leq 0.001$) from 3 other groups.

the other calcium groups when measuring Lat 3, but there was a trend towards faster completion of contraction with increasing amounts of calcium.

Figure 3. The effects of calcium on afterload performance parameters (means $\pm$ SEM) of turkey papillary muscle bioassays


LAT 1
LAT 2
LAT 3
LATENCY (mm)
WORK mgs-mm/CSA
REL VELOCITY mm/sec/CSA
CON VELOCITY mm/sec/CSA
Po (mg)
0
1.7
3.5
4.3
5.5
CALCIUM CONCENTRATION (Mm)

DISCUSSION

This work shows that turkey papillary muscles can be harvested, transected and remain functional for up to 6 hours in an *in vitro* chamber. Anatomically papillary muscles in the turkey heart are not as well developed as other species, i.e., cats, rabbits, and dogs. Nevertheless, preliminary studies showed that the harvest techniques which included quick transection will produce uniform muscle preparations.[17]

A modified K-H solution was developed that provides a suitable matrix for cardiac muscle function studies. Normal clinical pathology values of mature turkey plasma were available from the literature[20, 21] but were not found for young poults. Therefore, normal plasma biochemical values were determined for immature birds (Table 1) as a prerequisite to establishing the composition of a suitable K-H solution for muscle preparations from young poults. Several variables associated with K-H solutions influence isolated papillary muscle performance. These variables include temperature, pH, calcium ion concentration, and oxygenation.[18, 19, 22]

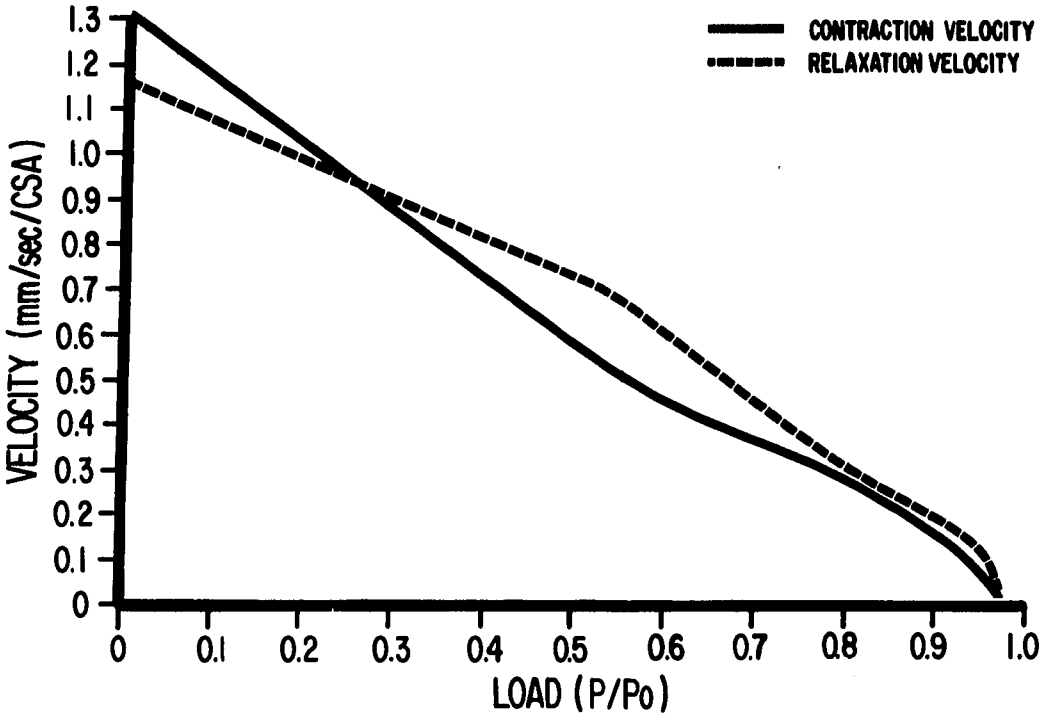
The effect of temperature on papillary muscle performance has been a source of controversy.[22, 23, 24, 25] The physiological temperature of turkeys is approximately 42° C suggesting that *in vitro* muscle performance might be ideal at similar temperatures. Our early studies comparing 37° C and 31° C showed that P_o and relaxation velocities were reduced at 37° C temperatures.[17] Papillary muscles studied at lower temperatures (31° C) may be responding to the Q_{10} or temperature coefficient involved

in the velocity of enzyme-catalyzed reactions. The velocity of many biological reactions roughly doubles with a 10° C rise in temperature. The effective operational temperature of 31° C prolongs the working life of an isolated muscle.

Turkey papillary muscle data compares well with other studies in our laboratory involving the dog.[19, 25, 26] Dog afterload velocity data were fit to 3rd power equations to establish V max and P_o values. Turkey afterload velocity data were best fit to a 3rd degree polynomial and Hill equations to determine V max values. A typical individual muscle afterload curve, demonstrating relaxation data fit to a 3rd degree polynomial and Hill equations is shown in Fig. 4. This curve exemplifies a frequently but unexplainable observation of a decreased relaxation V max when compared to contraction V max. This is also seen in the group mean values (Table 2). Muscle tension development undergoes depression with acidosis. In our laboratory, pH ranges that satisfactorily supported dog muscle preparations worked equally well for turkeys. K-H solution pH for turkeys closely correlates with physiological pH and other researchers' findings of an operational range of 7.25 - 7.60.[27, 28]

The ability of turkey papillary muscles to perform and function in K-H solutions with different calcium concentrations compared well with young dogs[19, 29] and other species.[4, 12] Mean P_o values for 1.7 and 3.4 mM calcium showed a linear, almost doubling relationship. The reduced P_o mean value for the 5.5 mM calcium group could be due to partial calcium toxicity. Normal turkey plasma values of total calcium are approximately 3.1 mM. Normal papillary muscle tissue concentrations of free or

Figure 4. Typical contraction and relaxation afterload curves generated by a turkey poult papillary muscle in 4.32 mM calcium. Data are fit to a third degree polynomial equation. The Hill equation was used for V max determinations


CONTRACTION VELOCITY
RELAXATION VELOCITY
VELOCITY (mm/sec/CSA)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
LOAD (P/Po)

unbound intracellular calcium would be more nearly approximated by the 1.7 mM concentration. This logic could partially explain the reason that experimental muscles in the 5.5, 4.3, and 3.5 mM calcium groups were observed to be slower to adapt to experimental conditions when initially placed in the *in vitro* chamber.

Recent studies have been concerned with chemical or drug effects on the dynamics of avian cardiac mechanics.[30, 31, 32] Our study demonstrates that the turkey papillary muscle evaluation system could be utilized to provide preliminary *in vitro* evaluation of chemicals and drugs prior to clinical trials, pathophysiological data, or intermediate correlations between cardiac cell cultures and *in vivo* application.

REFERENCES

1. Sonnenblick EH. Determinants of active state in heart muscle: force, velocity, instantaneous muscle length, time. Fed Proc 24:1396-1409, 1965.

2. Sheridan DJ. Postnatal developmental changes in the electrophysiological properties of cat right ventricular papillary muscles. Cardiovasc Res 14:700-709, 1980.

3. Cheng HC, Kariya T, Gleason EM, et al. Studies on the mechanism of the positive inotropic effect of piroximone in cat papillary muscle. J Cardiovasc Pharmacol 4:509-514, 1982.

4. Bosnjak ZJ, Kampine JP. Effects of halothane on transmembrane potentials calcium transients and papillary muscle tension in the cat. Am J Physiol 251:H374-381, 1986.

5. Hayashi H, Suzuki Y, Masumura Y, et al. Effects of L-carnitine on action potential of canine papillary muscle impaired by long chain acyl carnitine. Jpn Heart J 23:623-630, 1982.

6. Rouleau JL, Paradis P, Shenasa H, et al. Faster time to peak tension and velocity of shortening in right versus left ventricular trabeculae and papillary muscles of dogs. Circ Res 59:556-561, 1986.

7. Wisenbaugh T, Allen P, Cooper GIV, et al. Contractile function, myosin ATPase activity and isozymes in the hypertrophied pig left ventricle after a chronic progressive overload. Circ Res 53:332-341, 1983.

8. Binah O, Rubinstein I, Bromzon A, et al. Effects of bile acids on ventricular muscle contraction and electrophysiological properties: studies in rat papillary muscle and isolated ventricular myocytes. Arch Pharmacol 335:160-165, 1987.

9. Siegl PKS, Cragoe EJ, Trumble MJ, et al. Inhibition of Na^+/Ca^{2+} exchange in membrane vesicle and papillary muscle preparations from guinea-pig heart by analogs of amiloride. Proc Natl Acad Sci 81:3238-3242, 1984.

10. Wohlfart B. Analysis of mechanical alterans in rabbit papillary muscle. Acta Physiol Scand 115:405-414, 1982.

11. Komai H, Rusy BF. Negative inotropic effects of isoflourane and halothane in rabbit papillary muscles. Anesth Analg 66:29-33, 1987.

12. Marban E, Kusuoka H, Yue DT, et al. Maximal Ca^{2+}-activated force elicited by tetanization of ferret papillary muscle and whole heart: mechanism and characteristics of steady contractile activation in intact myocardium. Circ Res 59:262-269, 1986.

13. Rossner KL, Coudrai PR. Contractile properties of papillary muscle from cardiomyopathic hamsters: effects of isoprenaline. Cardiovasc Res 20:609-613, 1986.

14. Lewis RJ, Endean R. Direct and indirect effects of Ciguatoxin on guinea-pig atria and papillary muscles. Arch Pharmacol 334:313-322, 1986.

15. Kanaya S, Katzung BG. Effects of diltiazem on transmembrane potential and current of right ventricular papillary muscle of ferrets. J Pharmacol Exp Ther 228:245-251, 1984.

16. Lefer AM, Martin J. Mechanism of the protective effect of corticosteroids in hemorrhagic shock. Am J Physiol 216:314-320, 1969.

17. Miller CD, Hembrough FB, Terry WH. Development of a turkey papillary muscle assay system. Abstr Pap Annu Meet Conf Res Work Anim Dis 64:19, 1983.

18. Hill AV. The diffusion of oxygen and lactic acid through tissues. Proc R Soc Lond 104:39-96, 1928.

19. Hembrough FB, Anderson A, Tang R, et al. Potential of papillary muscle from young dogs for use in various diagnostic studies. Am J Vet Res 39:1936-1942, 1978.

20. Mitruka BM, Rawnsley HM. Clinical biochemical and hematological reference values in normal experimental animals. New York: Masson Publishing Inc., 143-144, 1977.

21. Freeman BM. Appendix: Biochemical and physiological data. In Physiology and Biochemistry of the Domestic Fowl. New York: Academic Press 5:412, 1984.

22. Kababgi MD, Schneider KW. Effect of temperature and calcium on the diastolic length-tension relationship of rat heart muscle. Acta Cardiologica 35:215-225, 1980.

23. Parmley WW, Chuck L, Sonnenblick E. Relation of V max to different models of cardiac muscle. Circ Res 30:34-43, 1972.

24. Johnson EA, Sonnenblick E, Ford L. Temperature. In The Physiological Basis of Starling's Law of the Heart. Porter R, Fitzsimons D. eds, Associated Scientific Publishers, New York 236-237, 1974.

25. Terry WH. Production and bioassay of an endogenous myocardial depressant. PhD dissertation, Iowa State University, Ames, Iowa 108-109, 1985.

26. Glassman R. Possible depression of canine papillary muscle contractility with plasma from aged dogs. Master's thesis, Iowa State University, Ames, Iowa, 1980.

27. Goldfarb RD, Weber P. Separation of pancreatic cardio-depressant activity into peptide and salt activity. Proc Soc Exp Biol Med 156:219-222, 1977.

28. Lefer AM, Blattberg B. Comparison of the effects of two factors present in plasma of shocked animals. J Reticuloendothelial Soc 5:54-60, 1968.

29. Hembrough FB, Miller CD, Williams K., et al. A technique developed for isolated canine papillary muscle function in various plasmas; and utility ascertained with plasma containing myocardial depressant factors. Am J Vet Res 41:700-706, 1980.

30. Lipshultz S, Shanfield J, Chacko S. Emergence of B-adrenergic sensitivity in the developing chicken heart. Proc Natl Acad Sci USA 78: 288-292, 1981.

31. Merril GF, Russo RE, Halper JM. Cardiac output distribution before and after endotoxin challenge in the rooster. Am J Physiol 241: R67-71, 1981.

32. Swayne DE, Shlosberg A, Davis RB. Salt poisoning in turkey poults. Avian Dis 30:847-852, 1986.

SECTION II. ALTERED FUNCTION OF TURKEY PAPILLARY MUSCLES EXPOSED TO CYCLOPIAZONIC ACID MYCOTOXIN

Altered Function of Turkey Papillary Muscles Exposed To Cyclopiazonic Acid Mycotoxin

C. D. Miller[1], DVM, MS, J. L. Richard[1], PhD, F. B. Hembrough[2], DVM, PhD, G. D. Osweiler[3], DVM, PhD, and D. F. Cox[4], PhD

[1]National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, P. O. Box 70, Ames, IA 50010.

[2]Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

[3]Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

[4]Department of Statistics, College of Science and Humanities, Iowa State University, Ames, IA 50011.

SUMMARY

An *in vitro* bioassay system was utilized to study the effects of cyclopiazonic acid mycotoxin on cardiac muscle. Acute exposure to CPA at 6 ug/ml significantly decreased five parameters of *in vitro* papillary muscle performance. The five performance criteria included; P_o, maximum contraction and relaxation velocity, time to peak contraction, and total time for muscle contraction and relaxation. These altered performances appear to be intracellular changes partially associated with calcium availability and were irreversible suggesting that physiopathological changes had occurred following acute CPA exposure.

INTRODUCTION

Cyclopiazonic Acid (CPA) is a mycotoxin produced by a number of the genera Aspergillus and Penicillium.[1] Studies indicate that CPA is quite prevalent in corn and peanuts; however, the potential mycotoxicological problem has not been established. Foods and feeds of both humans and animals have been shown to contain CPA.[2-5] Therefore, CPA represents a potential toxicological problem of agricultural and economic significance.

Among other physiological and pathological changes, CPA alters cardiac function. Rabbits exposed to 5, 7, and 10 mg/kg demonstrated tachycardia followed by bradycardia and terminal ventricular fibrillation. Negative inotropic effects in isolated frog ventricles were observed 30 sec after exposure to 20 ug/ml of CPA.[6] Purchase[7] reported hyaline or coagulative degeneration of rat myocardium following oral doses ranging from 36 mg/kg in males to 63 mg/kg in females. Other myocardial pathological effects following weekly CPA doses of 12 or 21 mg/kg in rats, have included cellular degenerative changes of enlarged pleomorphic nuclei with margination of chromatin.[8]

An in vitro biological assay system that utilizes turkey cardiac muscle was utilized to investigate CPA cardiac effects. The system was effective for measuring a battery of muscle performance criteria including muscle contraction and relaxation velocities, load values,

amplitudes and latencies.[9] This study was done to evaluate and characterize the functional effects of CPA on cardiac muscle through the utilization of this biological assay system.

MATERIALS AND METHODS

Turkey papillary muscle preparation Nine turkey poults (8-14 weeks old, Broad Breasted White) were utilized in this study. Each bird was anesthetized with sodium pentobarbital (65 mg/kg) just prior to tissue preparation. The heart was removed and left ventricular papillary muscles were cut to dimensions of 1 mm x 1 mm x 7-10 mm as previously described.[9]

Equipment Papillary muscles were mounted in the inner chamber of a double chambered constant-temperature bath. The mounting technique, apparatus and chamber fluid circulation was performed as previously reported.[9] The chamber fluid was a modified Krebs-Henseleit (K-H) solution containing: NaCl (118 mM); KCl (4.7 mM); $MgCl_2$ (1.2 mM); $Na_2H_2PO4 \cdot H_2O$ (11 mM); $NaHCO_3$ (24.0 mM); $CaCl_2 \cdot 6H_2O$ (1.7 mM); glucose (4.5 mM); pH 7.40. Chamber fluid was circulated by an individual rate controlled pump. The total system required 10 ml of fluid. The fluid was aerated with 95% O_2 - 5% CO_2, maintained at 31° C and 7.4 pH. Silver electrodes were arranged on each side of the muscle. The muscle was stimulated with 20 mVolts, at a frequency of 10 pulses per second, with 5 msec duration. Time, electrical stimulus, and muscle contractions were recorded with a 4-channel Beckman polygraph (No. 611, Beckman Instruments Inc., Electronic Instruments Division, Schiller Park, IL).

CPA toxin Purified crystalline CPA was produced according to previously described methods.[10] Purity was determined to be 95% by

thin-layer chromatographic and gas chromatographic mass spectral analyses conducted in another laboratory (R. J. Cole, USDA, ARS, National Peanut Research Laboratory, Dawson, GA). The CPA was dissolved in ethanol at a concentration so that 6 ul of solution contained the desired quantity of CPA to give a muscle chamber solution concentration of 6 ug/ml.

Contractile studies Muscles were stabilized for 2-2 1/2 hours while contracting isotonically in K-H solution. Following stabilization, the length of muscle that produced maximal contraction (L max) was determined for each muscle preparation. The procedures for determining L max and 90% L max were as previously described.[11]

Seven parameters of muscle function were obtained from experiments using the nine turkey papillary muscles. These parameters were: the maximum weight a muscle could lift (P_o); maximum contraction and relaxation velocities (V max); time delay or latency between electrical stimulation and initiation of contraction (Lat 1), peak contraction (Lat 2), or completion of contraction and relaxation (Lat 3) and distance a weight was lifted (Work). V max was determined by the Hill equation[12] from contraction or relaxation values generated by each afterload curve. Latency values were measured from recordings of contraction curves produced by the preload weight or the 90% of L max weight (where 25 mm = 1000 ms). Each set of muscle afterload data was normalized by length, cross-sectional area, and P_o for each muscle.

CPA solution studies The K-H solution was prepared on the experimental day. Following the completion of a 2 1/2 hour isotonic stabilization period and preload determination of each muscle, the K-H solution was changed. The order of muscle exposure to solutions was K-H solution (K-H), K-H solution with 6 ul ethanol (K-H + E), K-H solution with 6 ul ethanol plus 6 ug/ml CPA (K-H + E CPA), and a second K-H solution (K-H). The solution was changed following the completion of an afterload curve for each solution. The solution in the chamber and tubing was quickly drained and the next solution was added. During these solution changes, the muscle was maintained by bathing with K-H solution applied with a disposable pipette. The muscle was allowed to equilibrate in each solution for a 15 minute period before an afterload curve was generated.

Statistical analysis Afterload curve contraction and relaxation velocity data were described using a 3rd degree polynomial equation. The V max, P_o, Work and latency values were subjected to an analysis of variance that considered the muscle and the four exposures as sources of variation. Means were compared using a least significant difference ($P \leq 0.05$).

RESULTS

The effects of CPA on muscle performance are shown in Table 1. These afterload data indicate that there were significant differences among the means of all measured parameters. Mean comparisons for each group of the seven significant CPA-related effects are shown graphically in Figure 1. We found that mean comparisons of muscle performance in K-H + ethanol and K-H + ethanol and CPA showed five means that were significantly different. Those parameters were maximum contraction velocity, maximum relaxation velocity, P_o, latency 2 and latency 3. Means of latency 1 indicated a trend similar to latency 2 and 3 of slower completion of contraction while exposed to CPA.

There were no significant differences among mean comparisons for all afterload parameters, when comparing performance in K-H + ethanol and CPA and the second K-H solutions. Contraction and relaxation maximum velocities, and the three latency measurements showed trends toward returning to base line values of the initial K-H + ethanol solutions (Table 1, Figure 1). When comparing the muscle's ability to do work, a trend of steady decline in performance was observed in all solutions following the initial K-H.

In all instances, mean comparisons between the performance in initial K-H and K-H + ethanol solutions were not significantly different.

Figure 1. The effects of Cyclopiazonic acid on afterload parameters (means) of turkey papillary muscle bioassays. Experimental solutions are; K-H = Krebs-Henseleit, K-H+E = Krebs-Henseleit plus ethanol, K-H+E CPA = Krebs-Henseleit plus ethanol and Cyclopiazonic acid

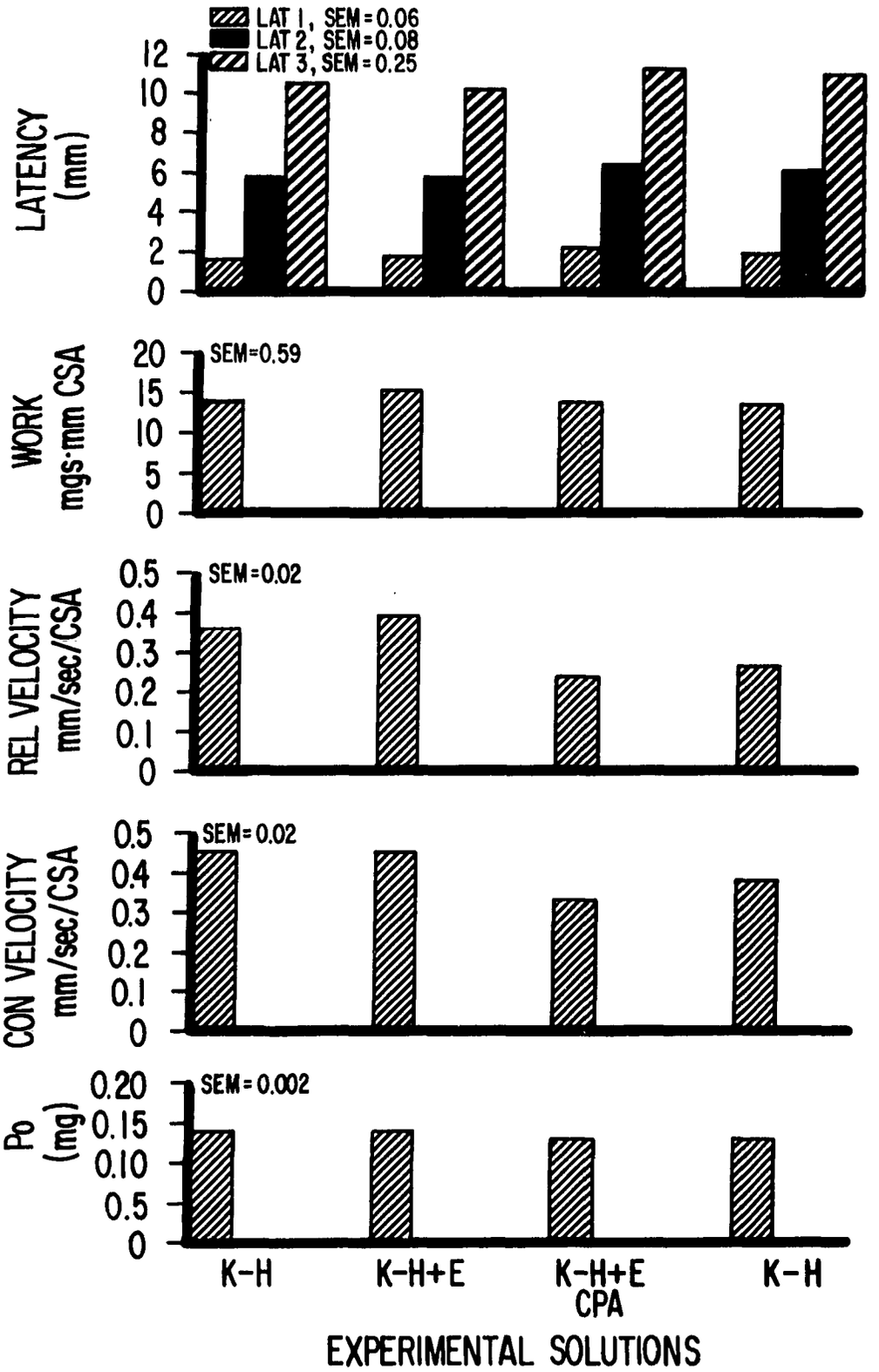

LAT 1, SEM = 0.06
LAT 2, SEM = 0.08
LAT 3, SEM = 0.25
LATENCY (mm)
12
10
8
6
4
2
0
WORK mgs·mm CSA
SEM=0.59
20
15
10
5
0
REL VELOCITY mm/sec/CSA
SEM=0.02
0.5
0.4
0.3
0.2
0.1
0
CON VELOCITY mm/sec/CSA
SEM=0.02
0.5
0.4
0.3
0.2
0.1
0
Po (mg)
SEM=0.002
0.20
0.15
0.10
0.5
0
K-H
K-H+E
K-H+E CPA
K-H
EXPERIMENTAL SOLUTIONS

Table 1. Mean contraction and relaxation velocities, P_o, work and latency afterload values of turkey papillary muscles in response to 6 ug/ml cyclopiazonic acid (CPA)

	Solutions and Sequence of Exposure				
	Krebs-Henseleit	Krebs-Henseleit Ethanol	Krebs-Henseleit Ethanol + CPA	Krebs-Henseleit	L.S.D. ($P \leq 0.05$)
Contraction					
Maximum Velocity (mm/sec/CSA)[a,b,c]	0.46*	0.46*	0.33+	0.38+	0.050
Relaxation					
Maximum Velocity (mm/sec/CSA)[a,b,c]	0.37*	0.39*	0.24+	0.27+	0.060
P_o (mg)[a,c,d]	0.14*	0.14*	0.12+	0.12+	0.002
Work (mgs.mm/CSA)[a,c]	16.70*	15.60*+	13.90+	13.50+	1.790
Latency 1 (mm)[c]	1.60*	1.70*+	2.20+†	2.00†	0.190
Latency 2 (mm)[c]	5.70*	5.70*	6.50+	6.20+	0.230
Latency 3 (mm)[c]	10.50*	10.20*	11.30+	11.00+	0.750

[a] Normalized for muscle cross sectional area (CSA).

[b] Maximum contraction and relaxation velocities are y intercepts, determined by plotting velocity vs load.

[c] The standard errors for the means are; Con Vel (0.02), Rel Vel (0.02), P_o (0.002), Work (0.59), Lat 1 (0.06), Lat 2 (0.08), Lat 3 (0.25).

[d] P_o is the maximum isometric weight load of a muscle.

*+† Means with the same symbol are not significantly different ($P \leq 0.05$).

DISCUSSION

This study demonstrates that the turkey papillary muscle evaluation system can be utilized to provide preliminary *in vitro* cardiac evaluation of the mycotoxin CPA.

Our data indicate that acute exposure to CPA at 6 ug/ml significantly decreased five parameters of muscle performance. The five performance criteria included; maximum contraction velocity, maximum relaxation velocity, P_o, latency 2 and latency 3. Cyclopiazonic acid significantly decreased both maximum contraction and maximum relaxation velocities. Combining these decreased velocities with the observed decreased P_o delays in time to peak contraction and total time for muscle contraction and relaxation suggests that the site of action is intracellular and calcium related. The onset of contraction can be altered by influencing the sarcolemma. Either the action potential or the transsarcolemmal pulse of trigger calcium necessary to cause calcium release by the sarcoplasmic reticulum could be altered. Decreased relaxation velocity can be attributed to impaired calcium uptake by the sarcoplasmic reticulum or slowed cross-bridge turn over. Calcium governs both the number of active cross-bridges between actin and myosin filaments during tension development and the rate of turn over of cross-bridge formation.[13-15] P_o represents the maximum isometric force and is proportional to the number of active actin-myosin sites capable of producing this force. Calcium exposes the myosin binding sites of the

actin filament and directly activates ATPase. Varied calcium concentrations have been shown to alter P_o.[9,16]

CPA is a lipophilic monobasic acid known to complex with divalent cations.[4] CPA alters either ion pumps or membrane permeability.[17] If CPA complexed with calcium as it entered the cell following depolarization of the cell membrane or altered cell permeability, the activation of the papillary muscle mechanical response would be greatly altered and could explain portions of the effects found in the present study.

The inability of the muscles to significantly recover from exposure to CPA was observed in all parameters. These results are strikingly different from previous work with T-2 mycotoxin and ethanol. Depressed muscle functions were restored after re-equilibration in K-H solution following exposure to T-2 toxin at either 2.5 or 7.5 ug/ml.[16] Muscle exposure to 0.6 ul/ml of ethanol did not significantly decrease muscle performance. These results are consistent with previous results where muscle performance was not significantly altered following two consecutive exposures to 0.6 ul/ml of ethanol over a similar time period.[16] This lack of recovery further suggests that the effects of CPA are the result of intracellular functional and/or morphological changes. These results are not fully explained by CPA calcium interaction but support previous work that demonstrated a continual degradation of <u>in vivo</u> cardiac function until terminal ventricular fibrillation occurred.[6]

Cyclopiazonic acid did not significantly alter muscle performance as measured by work and latency 1. However, there was a trend towards decreased performance in these measurements. We attribute part of this lack of statistical difference to measuring sensitivities that may not precisely reflect exact differences in latency.

REFERENCES

1. Richard JL, Peden WM, Fichtner RF, et al. Effect of cyclopiazonic acid on delayed hypersensitivity to Mycobacterium tuberculosis, complement activity, serum enzymes, and bilirubin in guinea pigs. Mycopathologia 96:73-77, 1986.

2. Orth R. Mycotoxins of Aspergillus oryzae strains for use in food industry as starters and enzyme producing molds. Ann Nutr Aliment 31:617-624, 1977.

3. Le Bars J. Cyclopiazonic acid production by Penicillium camemberti Thom and natural occurrence of this mycotoxin in cheese. Appl Environ Microbiol 38:1052-1055, 1979.

4. Gallagher RT, Richard JL, Stahr MH, et al. Cyclopiazonic acid production by aflatoxigenic and non-aflatoxigenic strains of Aspergillus flavus. Mycopathologia 66:31-36, 1978.

5. Lansden J, Davidson J. Occurrence of cyclopiazonic acid in peanuts. Appl Environ Microbiol 45:766-769, 1983.

6. Nishie K, Porter JK, Cole RJ, et al. Toxicity and neuropharmacology of cyclopiazonic acid. Food Chem Toxicol 23:831-839, 1985a.

7. Purchase IFH. The acute toxicity of the mycotoxin cyclopiazonic acid to rats. Toxicol Appl Pharmacol 18:114-123, 1971.

8. Van Rensburg SJ. Subacute toxicity of the mycotoxin cyclopiazonic acid. Food Chem Toxicol 22:993-998, 1984.

9. Miller CD, Hembrough FB, Richard JL, et al. In Vitro evaluation of isolated turkey cardiac muscle function. Vet Hum Toxicol 30:558-562, 1988.

10. Dorner JW. Production of cyclopiazonic acid by Aspergillus tamarii Kita. Appl Environ Microbiol 46:1435-1437, 1983.

11. Hembrough FB, Anderson A, Tang R, et al. Potential of papillary muscle from young dogs for use in various diagnostic studies. Am J Vet Res 39:1936-1942, 1978.

12. Brady A. Time and displacement dependence of cardiac contractility: Problems in defining the active state and force-velocity relations. Federation Proc 24:1410-1420, 1965.

13. Brutsaert DL, Claes V, Sonnenblick E. Velocity of shortening of unloaded heart muscle and the length tension relation. Circ Res 29:63-74, 1971.

14. Brutsaert DL, Claes V, Goethals M. Effects of calcium on force-velocity-length relationships of heart muscle of the cat. Circ Res 32:385-392, 1973.

15. Edman KA. The instantaneous force-velocity relationship as an index of the contractile state in cardiac muscle. Basic Res Cardiol 72:94-101, 1977.

16. Miller CD, Richard JL, Hembrough FB. In vitro evaluation of the cardioinhibitory properties of T-2 mycotoxin. Abstr Pap Annu Meet Conf Res Work Anim Dis 65:47, 1984.

17. Riley RT, Norred WP, Dorner JW, et al. Increased accumulation of the lipophilic cation tetraphenylphosphonium^{+} by cyclopiazonic acid-treated renal epithelial cells. J Toxicol Environ Health 15:779-788, 1985.

SECTION III. ULTRASTRUCTURAL AND FUNCTIONAL CHANGES OF TURKEY MYOCARDIUM INDUCED BY *IN VITRO* EXPOSURE TO CYCLOPIAZONIC ACID

Ultrastructural and Functional Changes of Turkey Myocardium Induced by *In Vitro* Exposure to Cyclopiazonic Acid

C. D. Miller[1], DVM, MS, G. D. Osweiler[2], DVM, PhD, J. L. Richard[1], PhD, D. F. Cox[3], PhD, and W. M. Peden[1], DVM, MS

[1]National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, P. O. Box 70, Ames, IA 50010.

[2]Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

[3]Department of Statistics, College of Science and Humanities, Iowa State University, Ames, IA 50011.

SUMMARY

Isolated papillary muscles were acutely exposed to 0 or 6 ug/ml of CPA mycotoxin. Seven parameters of *in vitro* papillary muscle performance were significantly decreased. The seven performance criteria were; P_o, work, maximum contraction and relaxation velocity, and times to initiation, peak, and completion of contractions. Calcium and ATP availability appear to be responsible, in part, for these altered performances. Electron photomicrographs demonstrated that CPA exposed muscles had increased numbers of swollen or lysed mitochondria.

This study provides additional evidence that at this exposure level, *in vitro* cardiac muscle performance is irreversibly decreased and mitochondrial morphology is altered.

INTRODUCTION

Cyclopiazonic Acid (CPA) is a mycotoxin produced by several fungal species in the genera Penicillium and Aspergillus. The toxicity of CPA has been experimentally demonstrated in rats, mice, rabbits, dogs, chickens, and pigs.[1-10] In vitro experiments utilizing CPA have included tissue preparations of esophagus, jejunum, ileum, trachea, aorta, vas deferens, uterus, ventricles, cell cultures of rat hepatocytes and pig renal epithelial cells, and sarcoplasmic reticulum vesicles from rat skeletal muscle.[11-15]

The effects of CPA on the ultrastructure of rat liver has been studied. Oral dosages of 2.0 and 4.0 mg/kg/day resulted in swollen mitochondria and vesiculation of the rough endoplasmic reticulum. Mitochondrial membranes were intact in all sections examined.[16] Ultrastructural changes were also reported in guinea pigs dosed at total doses of 4, 8, 12, and 16 mg/kg. Lower doses resulted in limited mitochondrial swelling. Animals that received 12 and 16 mg/kg showed a dose response in severity and degree of dilated sarcoplasmic reticulum, swollen mitochondria, and segmental necrosis in gastrocnemius and vastus lateralis musculature.[17] In monkeys exposed to levels of CPA up to 60 mg/kg/d for 16 weeks, ultrastructural changes were found in the capillaries of the heart and kidneys. Capillary endothelium showed irregular thickening and contained cytoplasmic vacuoles or large fenestrations. Cytoplasms had irregular processes or long irregular foldings. In myocytes focal lysis of myofibrils, mitochondrial

accumulation and lysosome formations were noted. Hepatocellular dialated rough endoplasmic reticulums were also observed.[18]

An earlier study demonstrated that CPA decreased turkey cardiac muscle performance following in vitro exposure to CPA.[19] This study was performed to further characterize the observed decrease in cardiac muscle performance and correlate physiological performance with pathological changes. Electron microscopy was utilized to assess muscle ultrastructural changes and determine sites of injury.

MATERIALS AND METHODS

Turkey papillary muscle preparation Ten turkey poults (8-14 weeks old, Broad Breasted White) were utilized in this study. Each bird was anesthetized with sodium pentobarbital (65 mg/kg) just prior to tissue preparation. The heart was removed and left ventricular papillary muscles were cut to dimensions of 1 mm x 1 mm x 7-10 mm as previously described.[20]

Equipment Papillary muscles were mounted in the inner chamber of a double chambered constant-temperature bath. The mounting technique, apparatus utilized and chamber fluid circulation was performed as previously reported.[20] The chamber fluid was a modified Krebs-Henseleit (K-H) solution containing: NaCl (118 mM); KCl (4.7 mM); $MgCl_2$ (1.2 mM); $Na_2H_2PO4 \cdot H_2O$ (11 mM); $NaHCO_3$ (24.0 mM); $CaCl_2 \cdot 6H_2O$ (1.7 mM); glucose (4.5 mM); pH 7.40. Chamber fluid was circulated by an individual rate controlled pump. The total system required 10 ml of fluid. The fluid was aerated with 95% O_2 - 5% CO_2, maintained at 31° C and 7.4 pH. Silver electrodes were arranged on each side of the muscle. The muscle was stimulated with 20 mVolts, at a frequency of 10 pulses per second with 5 msec duration and time, electrical stimulus, and muscle contractions were recorded with a 4-channel Beckman polygraph (No. 611, Beckman Instruments Inc., Electronic Instruments Division, Schiller Park, IL).

CPA toxin Purified crystalline CPA was produced according to previously described methods.[21] Purity was determined to be 95% by thin-layer chromatographic and gas chromatographic-mass spectral

analyses conducted in another laboratory (R. J. Cole, USDA, ARS, National Peanut Research Laboratory, Dawson, GA). The CPA was dissolved in ethanol at a concentration so that when 6 ul of solution was added to the muscle chamber solutions, a concentration of 6 ug/ml was obtained.

Contractile studies Muscles were stabilized for 2-2 1/2 hours while contracting isotonically in K-H solution. Following stabilization, the length of muscle that produced maximal contraction (L max) was determined for each muscle preparation. The procedures for determining L max and 90% L max were as previously described.[22]

Seven parameters of muscle function were obtained from experiments using turkey papillary muscles. These parameters were: the maximum weight a muscle could lift (P_o); maximum contraction and relaxation velocities (V max); time delay or latency between electrical stimulation and initiation of contraction (Lat 1), peak contraction (Lat 2), or completion of contraction (Lat 3) and distance a weight was lifted (Work). V max was determined by the Hill equation.[23] from contraction or relaxation values generated by each afterload curve. Latency values were measured from one weight that corresponded to the preload weight or 90% of L max weight. Latency values were also derived from the contraction curves. Each set of muscle afterload data was normalized by length, cross-sectional area, and P_o for each muscle.

CPA solution studies The K-H solution was prepared on the experimental day. Following the completion of a 2 1/2 hour isotonic stabilization period and preload determination of each muscle, the K-H

solution was changed. Muscles were randomly assigned to either a treatment group of 5 muscles or a control group of 5 muscles. The order of muscle exposure to solutions was K-H solution (K-H1), K-H solution with 6 ul ethanol (K-H + E) or K-H solution with 6 ul ethanol plus 6 ug/ml CPA (K-H + E CPA), and a second K-H solution (K-H2). The solution was changed following the completion of an afterload curve for each solution. The solution in the chamber and tubing was quickly drained and the next solution was added. During these solution changes, the muscle was maintained by bathing with K-H solution. The muscle was allowed to equilibrate in each solution for a 15 minute period before an afterload curve was generated.

Electron microscopy Muscles were fixed in situ following the completion of each experiment. Chamber K-H solution was drained and replaced with cold (4 °C) 2.5% glutaraldehyde fixative. Each muscle remained in glutaraldehyde for 2 hours under tension from the 90% of L max weight. Muscles were washed three times with 0.1 M cacodylate buffer. The fixed tissues were allowed to come to room temperature and further processed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated, and embedded in plastic (Medcast, Ted Pella, Inc., Tustin, CA). One micron sections of cross sectional and longitudinal myofibers were examined for histological changes. Thin sections were cut with an LKB Ultrotome (LKB Producter AB, Stockholm-Bromma, Sweden), stained with uranyl acetate and lead citrate. Ultrastructural changes were assessed in a Philips 410 Electron Microscope (N. V. Philips Gloeilampenfabrikien, Eindhoven, The Netherlands).

Statistical analysis Afterload curve contraction and relaxation velocity data were fit to a 3rd degree polynomial equation. Group means of V max, P_o, work, and latency values were compared by analysis of variance.

RESULTS

The effects of CPA on muscle performance indicate that there were significant differences among the means of all measured afterload parameters as shown in Table 1. Comparisons were considered significantly different at the $P \leq 0.05$ level. We found that mean comparisons of muscle performance in K-H1 ethanol and K-H + ethanol and CPA showed seven means that were significantly different. Those parameters were maximum contraction velocity, maximum relaxation velocity, P_o, work, latency 1, latency 2, and latency 3.

There were no significant differences among mean comparisons for all afterload parameters, when comparing performance in K-H + ethanol and CPA and the second K-H solutions. Contraction and relaxation maximum velocities, work, and latency 2 and 3 measurements showed trends toward returning to base line values of the initial K-H1 solution (Table 1). There were no significant differences among mean comparisons for any parameters, when comparing the 5 control muscles performance in K-H1, K-H + ethanol and K-H2.

Electron photomicrographs of muscles exposed to 0.6 ug CPA/ml bathing solution demonstrated mitochondrial changes when compared to control muscles. These mitochondrial changes were characterized by swelling (Figure 1) and cristoloysis, disorganization or lysis of cristae (Figures 2, 3). These changes were not uniformly observed throughout muscle preparations, but no pattern of occurrence was

Table 1. Mean contraction and relaxation velocities, P_o, work and latency afterload values of turkey papillary muscles (N=5) in response to (6 ug/ml) cyclopiazonic acid (CPA)

	Krebs-Henseleit 1	Krebs-Henseleit + Ethanol[e] + CPA	Krebs-Henseleit 2	L.S.D.
Contraction				
Maximum Velocity				
(mm/sec/CSA)[a,b,c]	0.54*	0.42+	0.40+	0.05
Relaxation				
Maximum Velocity				
(mm/sec/CSA)[a,b,c]	0.57*	0.36+	0.39+	0.07
P_o (mg)[a,c,d]	0.23*	0.21+	0.21+	0.01
Work (mgs mm/CSA)[a,c]	18.90*	15.50+	15.60+	2.36
Latency 1 (mm)[c]	0.80*	1.10+	1.10+	0.21
Latency 2 (mm)[c]	5.05*	5.85+	5.75+	0.46
Latency 3 (mm)[c]	8.73*	10.85+	10.68+	0.69

[a] Normalized for muscle cross sectional area (CSA).

[b] Maximum contraction and relaxation velocities are y intercepts, determined by plotting velocity vs load.

[c] Mean and individual S.E.M. are; Con Vel 0.02, Rel Vel. 0.02, P_o (0.01), Work (0.59), Lat 1 (0.06), Lat 2 (0.08), Lat 3 (0.25).

[d] P_o is the maximum weight a muscle could lift.

[e] Five control muscles were exposed to 6 ul of only ethanol in K-H; there were no significant differences from muscles performing in K-H alone.

*+† Means in each row with the same symbol are not significantly different ($P \leq 0.05$).

Figure 1. Myocardial mitrochondrial swelling from a papillary muscle exposed *in vitro* to 6 ug CPA/ml bathing solution.
x 14,040

Figure 2. Myocardial mitochondrial swelling and crystolysis from a papillary muscle exposed *in vitro* to 6 ug CPA/ml bathing solution. x 14,040

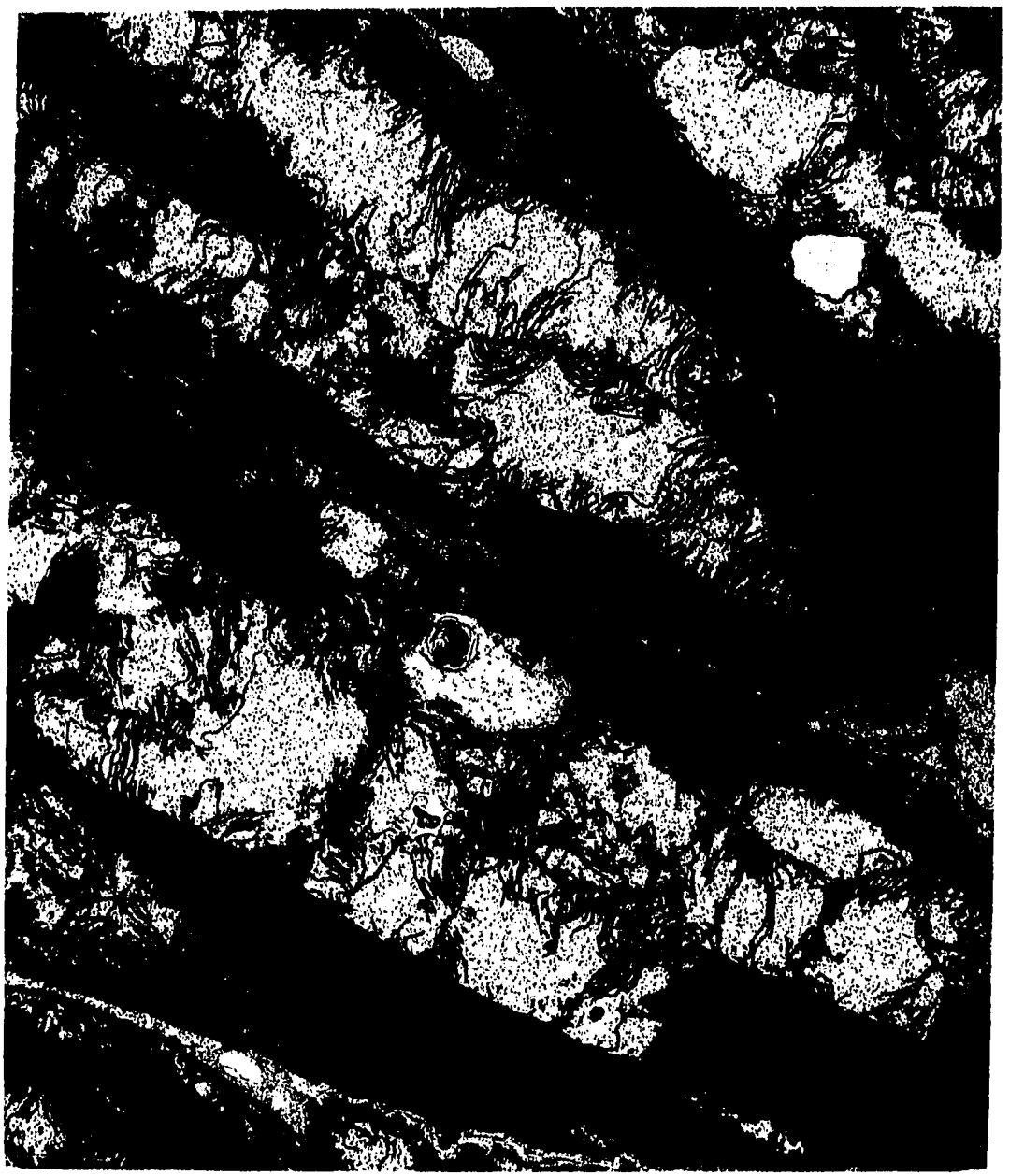


Figure 3. Myocardial mitochondrial disorganization or lysis of cristae from a papillary muscle exposed *in vitro* to 6 ug CPA/ml bathing solution. x 14,040

observed, i.e., muscle core vs peripheral. The transition from areas with normal mitochondria to affected areas was not gradual but succinct.

DISCUSSION

Acute exposure to CPA at 6 ug/ml significantly decreased all parameters of muscle performance. A previous study[19] demonstrated a significant ($P \leq 0.01$) decrease in five muscle performance parameters; maximum contraction velocity, maximum relaxation velocity, P_o, latency 2 and latency 3 following exposure to CPA. It was suggested, and further supported by this study, that the site of CPA action was intracellular and calcium related.[19]

Electron photomicrographs comparing the 5 control and 5 CPA treated muscles demonstrated that intracellular changes had occurred. These changes were restricted to mitochondria. Affected fibers showed swollen and lysed mitochondria. No other disruption of myofibril morphology was observed perhaps because this was an acute or subacute in vitro study. The altered mitochondrial morphology observed is difficult to interpret. Swollen mitochondria in liver and skeletal muscle has been previously reported following exposure to CPA.[16, 17] Mitochondria may be specifically damaged by a number of factors including, metabolic inhibition and deficiencies of electrolytes, enzymes or substrates. However, swollen mitochondria are associated with loss of capacity to synthesize ATP. A loss of ATP would also contribute to decreased muscle performance observed in this study.

The effects of altered intracellular calcium levels could also contribute to the significant decrease in both maximum contraction and maximum relaxation velocities and P_o. These results are consistent with

studies showing that CPA is a potent inhibitor of both calcium transport and calcium dependent ATPase activity of sarcoplasmic reticulum vesicles isolated from rat skeletal muscle.[15] Mitochondrial swelling and lysis could be explained by inhibition of calcium transport. Mitochondria accumulate calcium in a process that is energy coupled to electron transport. Electron transport energy can be used to accumulate calcium or to form ATP but not both.[24] Venoms toxic to muscle often act by decreasing the uptake and binding of calcium in mitochondria.[25, 26] Electrolyte composition disturbance by CPA could explain the mitochondrial swelling observed in this study. Mitochondrial swelling may occur by enhancement of cation influx and modification of the electrostatic barrier imposed by the phospholipid bilayer. This process involves water and metal salt influx through the inner mitochondrial membrane.[27]

REFERENCES

1. Purchase IFH. The acute toxicity of the mycotoxin cyclopiazonic acid to rats. Toxicol Appl Pharmacol 18:114-123, 1971.

2. Van Rensburg SJ. Subacute toxicity of the mycotoxin cyclopiazonic acid. Food Chem Toxicol 22:993-998, 1984.

3. Morrissey RE, Norred WP, Cole RJ, et al. Toxicity of the mycotoxin, cyclopiazonic acid, to sprague-dawley rats. Toxicol Appl Pharmacol 77:94-107, 1985.

4. Nishie K, Porter JK, Cole RJ, et al. Toxicity and neuropharmacology of cyclopiazonic acid. Food Chem Toxicol 23:831-839, 1985a.

5. Nishie K, Cole RJ, Dorner JW. Toxic effects of cyclopiazonic acid in early phase of pregnancy in mice. Res Commun Chem Pathol Pharmacol 53:303-315, 1987.

6. Neuhring LP, Rowland GN, Harrison LR, et al. Cyclopiazonic acid mycotoxicosis in the dog. Am J Vet Res 46:1670-1676, 1985.

7. Dorner JW, Cole RJ, Lomax LG, et al. Cyclopiazonic acid production by Aspergillus flavus and its effects on broiler chickens. Appl Environ Microbiol 46:698-703, 1983.

8. Wilson ME, Hagler WM, Ort JF, et al. Acute and subacute effects of cyclopiazonic acid in broiler chickens. Poultry Sci 65: 145, 1986.

9. Wilson ME, Hagler WM, Ort JF, et al. Subacute effects of cyclopiazonic acid in broiler chickens fed normal and high level dietary zinc. Poultry Sci 66:47, 1987.

10. Lomax LG, Cole RJ, Dorner JW. The toxicity of cyclopiazonic acid in weaned pigs. Vet Pathol 21:418-424, 1984.

11. Nishie K, Cole RJ, Dorner JW. Effects of cyclopiazonic acid on the contractility of organs with smooth muscles, and on frog ventricles. Res Commun Chem Pathol Pharmacol 53:23-37, 1986.

12. Riley RT, Goeger DE, Norred WP, et al. Age and growth-related changes in cyclopiazonic acid-potentiated lipophilic cation accumulation by cultured cells and binding to freeze-thaw lysed cells. J Biochem Toxicol 2:251-264, 1987.

13. Riley RT, Norred WP, Dorner JW, et al. Increased accumulation of the lipophilic cation tetraphenylphosphonium^{+} by cyclopiazonic acid-treated renal epithelial cells. J Toxicol Environ Health 15:779-788, 1985.

14. Riley RT, Showker JL, Cole RJ, et al. The mechanism by which cyclopiazonic acid potentiates accumulation of tetraphenylphosphonium in cultured renal epithelial cells. J Biochem Toxicol 1:13-29, 1986.

15. Goeger DE, Riley RT, Dorner JW, et al. Cyclopiazonic acid inhibition of the Ca^{2+}-transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles. Biochem Pharmacol 37:978-981, 1988.

16. Hinton DM, Morrisey RE, Norred RE, et al. Effects of cyclopiazonic acid on the ultrastructure of rat liver. Toxicol Lett 25: 211-218, 1985.

17. Peden WM. Effect of cyclopiazonic acid on guinea pig skeletal muscle. Abstr Pap Symp Cellular and Molecular Mode of Action of Selected Microbial Toxins in Foods and Feeds, Chevy Chase, MD, 1988.

18. Jaskiewicz K, Close PM, Thiel PG, et al. Preliminary studies on toxic effects of cyclopiazonic acid alone and in combination with aflatoxin B_1 in non-human primates. Toxicology 52:297-307, 1988.

19. Miller CD, Richard JL, Hembrough FB, et al. Altered function of turkey papillary muscles exposed to cyclopiazonic acid mycotoxin. Manuscript in preparation. National Animal Disease Center, Ames, IA.

20. Miller CD, Hembrough FB, Richard JL, et al. In Vitro evaluation of isolated turkey cardiac muscle function. Vet Hum Toxicol 30:558-562, 1988.

21. Dorner JW. Production of cyclopiazonic acid by Aspergillus tamarii Kita. Appl Environ Microbiol 46:1435-1437, 1983.

22. Hembrough FB, Anderson A, Tang R, et al. Potential of papillary muscle from young dogs for use in various diagnostic studies. Am J Vet Res 39:1936-1942, 1978.

23. Brady A. Time and displacement dependence of cardiac contractility: Problems in defining the active state and force-velocity relations. Federation Proc 24:1410-1420, 1965.

24. Alberts B, Bray D, Lewis J, et al., eds. In: Molecular Biology of the Cell. New York:Garland Publishing, 1983;497 pp.

25. Neeman I, Carlton GL, Burnett JW, et al. Purification of an endonuclease present in Crysaora quinquecirrha Venom. Proc Soc Exp Biol Med 166:374, 1981.

26. Kobayashi J, Nakamura H, Hirata Y, et al. Effects of venoms from Conidae on skeletal, cardiac and smooth muscles. Toxicon 20:823-830, 1982.

27. Cheville N, ed. In: Cell Pathology. Second ed. Ames:Iowa State University Press, 1983;122-124.

SECTION IV. THE TOXICITY OF CYCLOPIAZONIC ACID IN TURKEY POULTS: CLINICAL, PHYSIOLOGICAL AND SEROLOGIC OBSERVATIONS

The Toxicity of Cyclopiazonic Acid in Turkey Poults; Clinical, Physiological and Serological Observations

C. D. Miller[1], DVM, MS, J. L. Richard[1], PhD, G. D. Osweiler[2], DVM, PhD, D. F. Cox[3], PhD, and W. M. Peden[1], DVM, MS

[1]National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, P. O. Box 70, Ames, IA 50010.

[2]Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

[3]Department of Statistics, College of Science and Humanities, Iowa State University, Ames, IA 50011.

SUMMARY

This is the first report of cyclopiazonic acid toxicity in turkey poults. Acute exposure to CPA at 5, 10, and 20 mg/kg/d produced clinical symptoms, altered clinical pathology and gross lesions. Signs of disease included lethargy, ataxia, drooped head and wings, ruffled feathers, regurgitation, marked anorexia, decreased amount and unformed feces. There were decreases in total serum protein and albumin concentrations of poults in the two greater dose groups but no changes were found in serum concentrations of SDH, AST and CPK. Cyclopiazonic acid was found by thin-layer chromatography in extracts of the feces of all CPA-dosed turkey poults collected during the first 24 h period after initial dosing and corresponded to the dosage levels. The major gross lesions noted at necropsy occurred in the gastrointestinal tract and liver. Poults in the two greater dose groups had focal erosion of the proventriculus and ventriculus, a fibrino-gelatinous membrane present in the ventriculus, and increased liver weights.

INTRODUCTION

The mycotoxin cyclopiazonic acid (CPA), an indole tetramic acid with a molecular formula of $C_{20}H_{20}N_2O_3$, was first isolated from Penicillium cyclopium Westling.[1] Several fungal species in the genera Penicillium and Aspergillus produce CPA. Corn, peanuts, cheese and chicken meat have been shown to be contaminated with CPA.[2-6]

No information is available regarding the toxicity of CPA in turkeys; however, there are several studies of CPA toxicity in chickens. The ED_{50} of CPA for two, three and four day-old chicken embryos was found to be 2.4, 4.7 and 2.7 ug, respectively. The acute LD_{50} in day-old cockerels was found to be 21.71 mg/kg.[7] Another study determined a 72-hour LD_{50} of 12.0 mg/kg for male broiler chicks and 12.1 mg/kg for females.[8] Cole[9] retrospectively speculated that the acute effects, hemorrhagic enteritis lesions and neurological symptoms observed in "Turkey X disease"[10] that killed 100,000 turkey poults, were produced by CPA and not aflatoxin. Dose-related lowered feed conversion and decreased feed intake have been reported in chronic feeding studies with CPA in chickens.[8,11,12] Chickens dosed orally with 10 mg/kg lost weight within 24 h. By 96 h after dosing, body weight began to recover but birds were stunted compared to controls. Birds given 0.5 or 5.0 mg/kg gained weight, but at significantly slower rates than controls; the 10 mg/kg group exhibited a subdued behavior and appeared unthrifty.[6,13] Chicks gavaged with 2 or 4 mg/kg of CPA in corn oil had an increased plasma creatine kinase activity that was positively

correlated with skeletal muscle degeneration.[14] In a similar study, a dose-related decrease in hematocrit and plasma potassium and protein was noted in chicks dosed with CPA at 1, 2 or 4 mg/kg for 23 days.[8] Deaths occurred at the higher doses in chickens fed CPA at 10, 50, and 100 ppm for 7 weeks.[11] Post mortem examination of the birds given 100 ppm revealed areas of erosion, ulceration and hyperemia of the proventricular mucosa. There also was excess mucus overlying the mucosal surface and dilation of the proventricular lumen. Birds given 50 ppm had dilation of proventricular lumens and apparent thickened proventricular mucosal surfaces. Gross lesions were not observed in birds given 10 ppm. Similar histologic lesions of necrosis and hemorrhage or hyperplasia of the proventricular mucosa was reported in chicks given CPA at 1, 2 or 4 mg/kg.[14]

The purpose of this study was to characterize the clinical signs, gross lesions and alterations in serum chemistry associated with oral administration of CPA to turkey poults.

MATERIALS AND METHODS

Animals Twenty, 10-wk-old, broad-breasted, white turkeys (Midwest Hatchery, Dike, IA) were used in this study. They were individually caged and fed (NADC Turkey Starter, Ration #517, Purina Mills Inc., St. Louis, MO) and watered ad libitum.

CPA mycotoxin Purified crystalline CPA was produced according to previously described methods.[11] Purity was determined to be 95% by thin-layer chromatographic and gas chromatographic mass spectral analysis conducted in another laboratory (R. J. Cole, USDA, ARS, National Peanut Research Laboratory, Dawson, GA). The CPA was weighed into No. 5 gelatin capsules for oral dosing and amounts were based on daily bird weights.

Experimental design Turkeys were randomly assigned to individual cages and 4 groups of 5 turkeys each at dosages of 0, 5, 10, and 20 mg/kg/d of CPA toxin. The birds were acclimated for 10 days before dosing began. Each poult was dosed daily for 2 days with a balling gun. A ten ml blood sample was obtained just prior to dosing and at end of the experiment. Blood samples were centrifuged and serum was removed for storage at -70° C. Fecal material was collected from each bird for the first 24 hours and extracted for CPA analysis according to previously described methods.[15] Briefly, 100 gm of feces was mixed with 25 gm of diatomaceous earth and 200 ml of distilled H_2O before acidification with 2 ml of 12N HCL. This mixture was extracted with 300 ml $CHCL_3$ by shaking for 1 h at 300 rpm. Extracts were recovered by

filtering through fluted filter paper collecting only the $CHCl_3$ layer. The emulsion layer was washed with 100 ml $CHCl_3$ and this $CHCl_3$ was added to the previous extract. Combined extracts were filtered through #1 Whatman paper into evaporation flasks and evaporated to dryness under vacuum. The residue was transferred quantitatively to a 2-dram vial with $CHCl_3$, the solvent was evaporated to dryness under flowing nitrogen at 50° C and samples were stored at -80° C. Prior to thin-layer chromatographic (TLC) analysis samples were reconstituted in $CHCl_3$. Two spiked samples, a control, and a feed sample were also extracted. Birds were weighed, necropsied and organs weighed 48 hours after administration of the initial dose.

Fecal CPA analysis Extracted samples were analyzed by TLC for the presence of CPA. These analyses were performed on precoated silica gel 60 F-254 plates (5 by 10 cm; EM Laboratories, Inc., Elmsford, NY). Internal and external standards of CPA were included on each plate. Plates were developed (20 cm) in ethyl acetate, methanol, ammonium hydroxide (85-10-10), sprayed with dimethyl-amino-benzyaldehyde in ethanol (2% solution), dried, sprayed with 50% ethanolic sulfuric acid, and heated to 110° C for 20 min. CPA was detected visually as a blue spot and the quantity determined by visual comparison with the standard series.

Clinical pathology Serum sorbitol dehydrogenase (SDH), aspartate amino transferase (AST), creatine phosphokinase (CPK), albumin (Al) and total protein (TP) were determined using a centrifical analyzer (Rotachem IIa centrifical analyzer, Travenol Labs, Inc., Deerfield, IL).

Statistical analysis Group means of body weight, liver and spleen weights, feed consumption, fecal CPA content, serum Al and TP were compared by analysis of variance.

RESULTS

Clinical observations Clinical signs developed within 24 h after initial dosing in the turkey poults given CPA at 10 and 20 mg/kg/d. These were lethargy, ataxia, drooped head and wings, ruffled feathers, regurgitation, anorexia, and unformed plus decreased amounts of feces. Inactivity progressed to recumbency. Poults in the 20 mg/kg/d group were killed *in extremis* 30 h after initial dosing. All poults in the 10 mg/kg/d group appeared weak and lethargic the final 24 hours before death.

The difference in rate of body weight gain between control and the 2 greatest dosage groups was significant (Table 1, Fig. 1). Turkeys given 5 mg/kg/d appeared healthy and there was no significant difference in rate of body weight gain when compared to controls.

Feed intake was reduced with increasing doses of CPA. Reduced feed consumption was significantly obvious in the 20 and 10 mg/kg/d groups by 24 h and was more marked by 48 h. An average of only 7.2 mg of feed was consumed by the 20 mg/kg/d group during the experiment (Table 1). There was no significant difference in feed consumption between the control and 5 mg/kg/d groups.

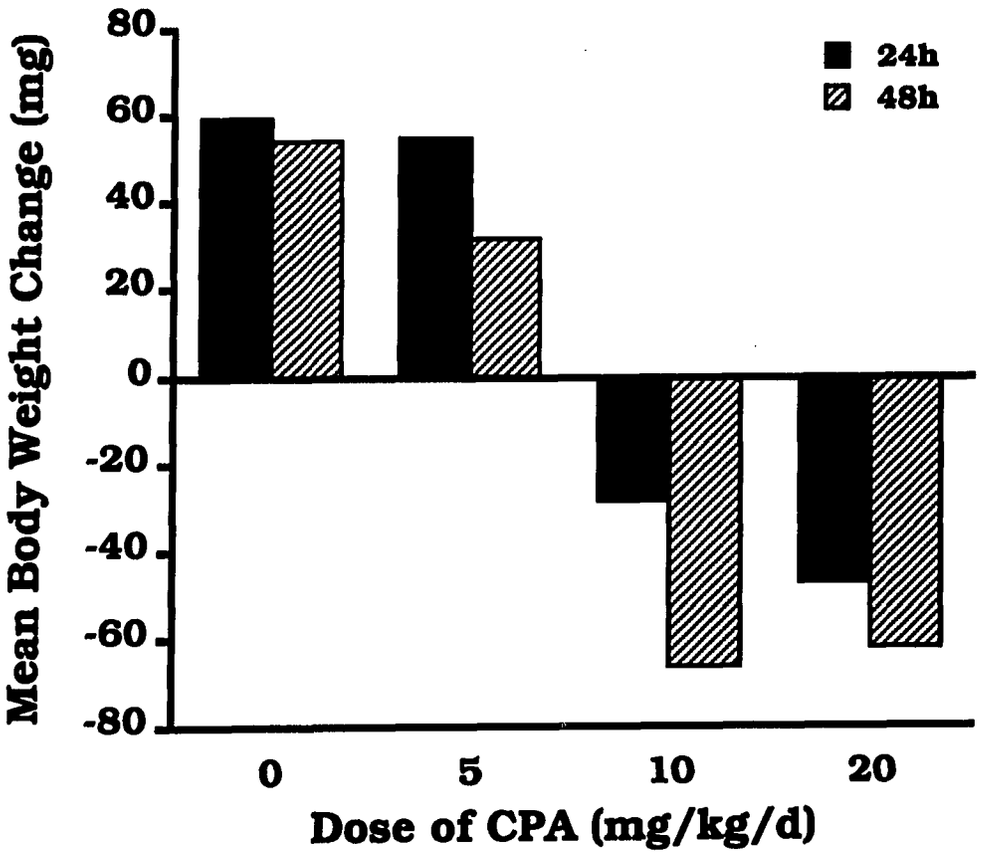
Gross lesions and organ weights At post mortem examination, poults given 10 and 20 mg CPA/kg/d had focal erosions of the proventriculus and ventriculus plus the presence of a diptheritic fibrinogelatinous membrane in the ventriculus. Poults given the highest doses had duodenal focal necrosis, enlarged gall bladders and 7 of 10 birds

Table 1. Mean body weight (bw), feed intake, liver, and spleen weight of turkey poults given oral cyclopiazonic acid

	Dosage Group (mg/kg/d)				
	0	5	10	20	L.S.D.
24 h Body Weight Change(g)	59.60*	55.40*	-28.20†	-46.60+	33.70
48 h Body Weight Change(g)	54.80*	32.40*	-66.20†	-61.80†	27.20
24 h Feed Intake (g)	119.40*	107.00*	24.60†	7.00†	20.40
48 h Feed Intake (g)	85.20*	80.40*	3.00†	0.20†	10.00
Liver Weight (% bw)	2.10*	2.46*$	2.84†$	3.00†	0.44
Spleen Weight (% bw)	0.10*	0.11*	0.09*	0.09*	0.05

*†$Means with the same symbol are not significantly different.

Figure 1. Change in the means of body weight (mg) of turkey poults (5/dosage group) given oral CPA. The 2 highest dosage groups were significantly different from the 5 mg/kg/d and control groups ($p < 0.05$)


80
60
40
20
0
-20
-40
-60
-80
Mean Body Weight Change (mg)
24h
48h
0
5
10
20
Dose of CPA (mg/kg/d)

had bloody and watery colon contents. Gross lesions were not found in either the 5 mg/kg/d or the control groups.

Liver weights expressed as a percentage of body weight showed a dose-related trend (Table 1). Significant differences were noted when comparing the control group to the 10 and 20 mg/kg/d groups. There was no significant differences when comparing the control and 5 mg/kg/d groups or the 5 and 10 mg/kg/d groups. Spleen weights were not significantly different among any of the treatment groups.

Fecal CPA analysis Fecal CPA content increased with each increase in dose. When comparing the 20 and 10 mg/kg/d groups, the increases were significantly different with fecal CPA content of the 20 mg/kg/d group twice that of the 10 mg/kg/d group (Table 2). The amounts of CPA in the feces of the 10 and 5 mg/kg/d groups were also significantly different. There was no significant difference between the 0 and 5 mg/kg/d; however, as expected, CPA was not present in untreated poults' feces.

Serum proteins and enzyme analysis Total serum protein and albumin concentrations in serum were decreased by increasing doses of CPA given to the poults. Significant ($P < 0.05$) decreases were noted at the 10 and 20 mg/kg/d levels when compared to controls and the 5 mg/kg/d groups. Serum SDH, AST and CPK concentrations were not significantly changed (Table 2).

Table 2. Mean albumin, total protein, fecal cyclopiazonic acid (CPA) content of turkey poults given oral CPA

	Dosage Group (mg/kg/d)				L.S.D.
	0	5	10	20	
Albumin (g/100 ml)	1.08*	1.03*	0.60†	0.68†	0.17
Total Protein	3.48*	3.10*	1.68†	1.88†	0.45
Fecal CPA Content (ug/g)	0*	3.60*	16.80†	34.30$	6.00

*†$Means with the same symbol are not significantly different.

DISCUSSION

Acute exposure to CPA produced clinical signs within 24 hrs of initial dosing in the 10 and 20 mg/kg/d groups. These clinical signs of lethargy, decreased weight gain, and decreased feed intake were similar to those previously reported in chickens[6,11] Ataxia, regurgitation and unformed feces due to CPA ingestion has not been previously described in birds. The regurgitation, decreased feed intake, and weight loss could all be partially related to the lesions present in the ventriculus. The diptheritic membrane present in all (10/10) poults in the 10 and 20 mg/kg/d groups was extensive enough to partially prevent deglutition. Death was imminent in the 20 mg/kg/d group and, therefore, were killed at 30 h post dosing. All of the birds were recumbent, and had not eaten for at least 12 h prior to the time they were killed. Cyclopiazonic acid was found in feces collected during the first 24 h period and corresponded in a linear relationship to the dosage levels. Apparently, following dosing, significant amounts passed through the bird to the feces. This is in agreement with Norred and coworkers[16] finding's that radio-labeled CPA was readily absorbed from the gut, its major route of excretion was fecal, and it had a half life of 43 $\pm$ 12 hrs following administration.

Relatively few gross lesions have been observed in animals following exposure to CPA. Chickens fed 50 or 100 ppm had lesions in the gastrointestinal tract.[11] Thickened mucosa and dilated lumens were noted in the proventriculi of birds fed 50 ppm dietary CPA. Birds from

the 100 ppm group had proventricular mucosal erosion and hyperemia.[11] Similar gross lesions including; hemorrhage, necrosis and hyperplasia of proventricular mucosa were reported in chickens gavaged with 1, 2 or 4 mg/kg CPA.[14] Focal erosions present in the proventriculus and ventriculus in our turkey poults given 10 and 20 mg CPA/kg/d were similar to lesions previously described. The presence of a fibrinogelatinous diptheritic membrane in the ventriculus may represent a local response to exposure to relatively high levels of toxin. Because this was an acute study, lesion sequella, i.e., thickened proventricular mucosa reported in more chronic studies, would not have had time to manifest themselves.

Wilson et al.[8] reported some changes in clinical pathological values in chickens following CPA exposure. In chicks dosed with CPA at 1, 2 or 4 mg/kg for 23 days, a dose-related decrease in plasma potassium and protein was noted. In the present study decreased total serum protein and albumin was observed in the 10 and 20 mg/kg/d groups. Duncan and Prasse[17] state that hypoproteinemia may be caused by hemorrhage, loss in urine, production loss, or intestinal loss. Some blood was present in the feces of poults in this study but no frank hemorrhage was observed. Amounts of protein lost in the urine/feces is unknown. Decreased production appears unlikely because, this was an acute, 30-48 h study and SDH levels were not decreased indicating the liver was functional. Therefore, intestinal loss appears to be the most likely explanation of decreased total serum protein and albumin. The fibrinogelatinous diptheritic membrane in the ventriculus and upper duodenum

represent protein loss that could account for these decreases. In cases of protein loss from serum, albumins are the smallest and first protein to move across the intestinal wall with globulins and fibrin crossing later.[18] In this study, albumin levels were decreased and corresponded with increased dose.

REFERENCES

1. Holzapfel CW. The isolation and structure of cyclopiazonic acid, a toxic metabolite of Penicillium cyclopium Westling. Tetrahedron 24:2109-2119, 1968.

2. Gallagher RT, Richard JL, Stahr MH, et al. Cyclopiazonic acid production by aflatoxigenic and non-aflatoxigenic strains of Aspergillus flavus. Mycopathologia 66:31-36, 1978.

3. Lansden J, Davidson J. Occurrence of cyclopiazonic acid in peanuts. Appl Environ Microbiol 45:766-769, 1983.

4. Still P, Eckardt C, Leistner L. Bildung von cyclopiazonsaure durch Penicillium camemberti-isolate von kase. Fleischwirtschaft 58: 876-877, 1978.

5. Le Bars J. Cyclopiazonic acid production by Penicillium camemberti Thom and natural occurrence of this mycotoxin in cheese. Appl Environ Microbiol 38:1052-1055, 1979.

6. Norred WP, Porter JK, Dorner JW, et al. Occurrence of the mycotoxin cyclopiazonic acid in meat after oral administration to chickens. J Agric Food Chem 36:113-116, 1988.

7. Vesley D, Vesela D, Neumannova V. Cyclopiazonic acid production: its effects on chicken embryos and day-old cockerels. Vet Med 30:345-352, 1985.

8. Wilson ME, Hagler WM, Ort JF, et al. Acute and subacute effects of cyclopiazonic acid in broiler chickens. Poultry Sci 65: 145, 1986.

9. Cole RJ. Etiology of turkey "x" disease in retrospect: a case for the involvement of cyclopiazonic acid. Mycotoxin Res 2:3-7, 1986.

10. Blount WP. Turkey "x" disease. Turkeys 9:52, 1961.

11. Dorner JW, Cole RJ, Lomax LG, et al. Cyclopiazonic acid production by Aspergillus flavus and its effects on broiler chickens. Appl Environ Microbiol 46:698-703, 1983.

12. Wilson ME, Hagler WM, Ort JF, et al. Subacute effects of cyclopiazonic acid in broiler chickens fed normal and high level dietary zinc. Poultry Sci 66:47, 1987.

13. Porter JK, Norred WP, Cole RJ, et al. Neurochemical effects of cyclopiazonic acid in chickens. Proc Soc Exp Biol Med 187:335-340, 1988.

14. Cullen JM, Wilson M, Hagler WM, et al. Histologic lesions in broiler chicks given cyclopiazonic acid orally. Am J Vet Res 49:728-731, 1988.

15. Norred WP, Cole RJ, Dorner JW, et al. Liquid chromatographic determination of CPA in poultry meat. J Assoc Off Anal Chem 70:121-123, 1987.

16. Norred WP, Morrissey RE, Riley RT. Distribution, excretion and skeletal muscle effects of the mycotoxin [^{14}C] cyclopiazonic acid in rats. Food Chem Toxicol 23:1069-1076, 1985.

17. Duncan JR, Prasse KW, eds. In: Veterinary Laboratory Medicine Clinical Pathology. Ames:Iowa State University Press, 1977; 21, 89-91, 125.

18. Kaneko JJ. Serum proteins and the dysproteinemias. In: Kaneko, JJ, ed. Clinical Biochemistry of Domestic Animals. 3rd Ed., Academic Press, New York, 1980;111.

SECTION V. PATHOLOGICAL AND HEMATOLOGICAL CHANGES IN YOUNG TURKEY POULTS ORALLY EXPOSED TO CYCLOPIAZONIC ACID

Pathological and Hematological Changes in Young Turkey Poults Orally Exposed to Cyclopiazonic Acid

C. D. Miller[1], DVM, MS, J. L. Richard[1], PhD, G. D. Osweiler[2], DVM, PhD, L. D. Miller[3], DVM, PhD, V. P. Meador[1], DVM, PhD, and T. L. Carson[2], DVM, PhD

[1]National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, P. O. Box 70, Ames, IA 50010.

[2]Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

[3]Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

SUMMARY

Effects of cyclopiazonic acid (CPA) on feed intake, water intake, hematological values and microscopic lesions were evaluated. Three groups of turkey poults, each containing 8 poults, were given either 0, 5, or 10 mg CPA/kg/d orally for 2 days. Poults given 10 mg CPA/kg/d had a significant ($P < 0.05$) decrease in body weight, feed consumption and water intake when measured at 24 and 48 hours. Turkeys given 10 mg/kg had significant hematological changes which included leukocytosis, heterophilia and lymphocytosis. Histopathologic changes consisting of mucofibrinous necrotizing proventriculitis and ventriculitis, multifocal ulcerative enteritis, and multifocal necrotizing hepatitis occurred in all poults dosed with 10 mg CPA/kg/d.

INTRODUCTION

Cyclopiazonic acid is a mycotoxin produced by several species in the genera Aspergillus and Penicillium. The extent of CPA contamination of food and feed has not been fully evaluated; however, corn, peanuts, cheese and millet have been shown to be naturally contaminated with CPA.[1-4]

The role of CPA in animal and human disease has not been established. The toxicity of CPA has been demonstrated in several animal species including rats, mice, guinea pigs, chickens, dogs, rabbits, pigs and monkeys.[5-31] Experimental toxicosis is predominantly characterized by gastrointestinal ulceration, splenic atrophy and necrosis, nephropathy, and hepatic and myocardial necrosis.

There appears to be species differences following oral exposure to CPA. These differences are dose sensitivity and affected target organs. In laboratory animals, the most commonly reported lesions include; hepatic vacuolation and necrosis, renal tubular nephrosis, splenic hemorrhage and necrosis, skeletal muscle degeneration and myocardial hyaline degeneration.[5-8,19,21] In pigs and chickens, gastrointestinal hyperemia, mucosal hyperplasia, ulceration and necrosis were reported in addition to lesions similar to those noted in laboratory animals.[22,24,30] In orally exposed monkeys mild renal tubular atrophy with protein casts was noted.[31] Dogs[28] are the most sensitive species tested for toxicity to CPA. In feeding studies, the major dose-related microscopic lesions were dermal necrosis, infarcts and ulceration and necrosis of the

gastrointestinal tract. Splenic lymphoid depletion and necrosis was also reported.

Several studies have reported lesions in the heart. Purchase[5] first reported cardiac lesions of hyaline or coagulative degeneration of rat myocardium following CPA exposure to oral doses ranging from 30 to 82.6 mg/kg. In chickens fed 50 and 100 ppm dietary CPA on an *ad libitum* basis, there was subacute to chronic inflammation of the myocardium noted at both dosing levels.[22]

Weekly dosages of 12 or 21 mg/kg in rats produced additional pathological changes of the myocardium which included cellular degenerative changes of enlarged pleomorphic nuclei, with margination of chromatin.[7]

Myocardial lesions were present in two monkeys treated with CPA; doses up to 60 mg/kg caused focal degenerative changes of focal atrophy and intracellular organelle disruption.[31] In contrast, no heart lesions were noted in CPA studies where oral doses were 0.1 or 4 mg/kg in rats,[14] 1, 2, or 4 mg/kg in broiler chicks,[24] 0.05, 0.25, 0.5, or 1.0 mg/kg in dogs,[28] and 0.01, 0.1, 1.0, or 10 mg/kg in piglets.[30]

Few studies have reported hematological observations. Hill *et al.*[12] reported that in rats given 0.1, 1.0 and 5.0 mg CPA/kg for 28 days, there was higher mean neutrophil counts and lower mean eosinophil and lymphocyte counts than in controls. Packed cell volume was unaltered. Cyclopiazonic acid did not affect the degree of cellularity, myeloid to erythroid ratio and presence of all stages of maturation in bone marrow smears. Dose-related hematological changes in dogs dosed

at 0.05, 0.5 and 1.0 mg/kg, consisted of leukocytosis, neutrophilia, lymphopenia, and monocytosis.[28]

The purpose of this study was to characterize pathological and hematological changes and to further characterize previously observed dehydration that we[32] associated with oral administration of CPA to turkey poults.

MATERIALS AND METHODS

Animals Twenty-four, 10-wk-old, broad-breasted, white turkeys (Midwest Hatchery, Dike, IA) were used in this study. They were individually caged and fed (NADC Turkey Starter, Ration #517, Purina Mills Inc., St. Louis, MO) and watered ad libitum.

CPA mycotoxin Purified crystalline CPA was produced according to previously described methods.[22] Purity was determined to be 95% by thin-layer chromatographic and gas chromatographic mass spectral analysis conducted in another laboratory (R. J. Cole, USDA, ARS, National Peanut Research Laboratory, Dawson, GA). The CPA was weighed into No. 5 gelatin capsules for oral dosing and amounts were based on daily bird weights.

Experimental design Turkeys were randomly assigned to individual cages and 3 groups of 8 turkeys each at dosages of 0, 5, and 10 mg/kg/d of CPA toxin. The poults were acclimated for 10 days before dosing began. Each poult was dosed daily for 2 days with a balling gun. Feed consumed and water intake was measured daily. Poults were weighed, blood samples were collected, and the poults were necropsied 48 hours after administration of the initial dose. Prepared blood smears were stained with the Dif-Quick method (American Scientific Products, McGaw Park, IL 60085) for differential counts. Total WBC counts were determined on fresh whole blood with the Unopette Test 5877 (Becton-Dickinson and Company, Rutherford, NJ 07070).

Histopathology Portions of liver, heart, spleen, brain, cervical spinal cord and gastrointestinal tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 um, stained with hematoxylin and eosin (H & E) stain and examined by light microscopy.

Electron microscopy Cardiac muscle tissue was fixed with cold (4° C) 2.5% gluteraldehyde for 2 hours. Muscles were washed three times and refrigerated in 0.1 M cacodylate buffer. The fixed tissues were allowed to come to room temperature and further processed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated, and embedded in plastic (Medcast, Ted Pella, Inc., Tustin, CA). One micron sections of cross sectional and longitudinal myofibers were examined for histological changes. Thin sections were cut with an LKB Ultrotome (LKB Producter AB, Stockholm-Bromma, Sweden) and stained with uranyl acetate and lead citrate. Ultrastructural changes were assessed from images using a Philips 410 Electron Microscope (N. V. Philips Gloeilampenfabrikien, Eindhoven, The Netherlands).

Statistical analysis Group means of body weight, feed consumption, water consumption, and hematological differential counts were compared by analysis of variance.

RESULTS

Clinical signs Clinical signs, similar to those described in an earlier study,[32] developed in the turkey poults given CPA at 10 mg/kg/d. These were lethargy, ataxia, drooped head and wings, ruffled feathers, regurgitation, anorexia, dehydration and decreased amounts of unformed feces. All poults in the 10 mg/kg/d group appeared weak and lethargic during the final 24 hours before death. Turkeys given 5 mg/kg/d appeared healthy. Body weight, feed consumption and water intake was significantly reduced in poults given 10 mg CPA/kg when measured at 24 and 48 h (Table 1). There were significant reductions in feed intake and body weight by 48 h in the 5 mg/kg/d group.

Hematological findings The 10 mg/kg dose group had significantly altered leukograms which had higher mean leucocyte, heterophil and lymphocyte counts than did the 5 mg/kg/d dose group or controls (Table 2). The 5 mg/kg/d group had a higher mean heterophil count as compared to controls, but the difference was not significant.

Microscopic lesions Microscopic lesions were limited to the liver and gastrointestinal tract of treated birds. No changes were found in heart, spleen, brain, or spinal cord tissues. Microscopic gastrointestinal lesions were present in all poults (8/8) in the 10 mg/kg/d group. They consisted of a mucofibrinous necrotizing proventriculitis (Figure 1) and ventriculitis (Figure 2). Lesions were accompanied by mucosal and submucosal edema and hemorrhage (Figures 3, 4), and swollen ventricular glandular cells. Diffuse but multifocally prominent

Table 1. Mean body weight (bw), feed intake, and water intake of turkey poults given oral cyclopiazonic acid for 2 days

	Dosage Group (mg/kg/d			L.S.D.
	0	5	10	
24 h body weight change(g)	62.8*	71.8*	-69.3$	27.3
48 h body weight change(g)	72.4*	1.6†	-134.1$	65.6
24 h feed intake (g)	140.8*	142.0*	9.4†	29.3
48 h feed intake (g)	177.3*	117.8†	7.6$	50.2
24 h water intake (g)	426.4*	456.6*	160.0†	78.0
48 h water intake (g)	582.1*	455.5*	105.6†	130.2

*†$Means with the same symbol are not significantly different.

Table 2. Mean hematological values of turkey poults given oral cyclopiazonic acid for 2 days

Item	Dosage Group (mg/kg/d) 0 (n=8)	5 (n=8)	10 (n=7)
WBC (cell x 10^{-2}/ul)[a]	278 ± 60*	290 ± 60*	870 ± 64†
Lymphocytes (cell x 10^{-2}/ul)[a]	132 ± 23*	103 ± 23*	223 ± 24†
Heterophils (cell x 10^{-2}/ul)[a]	130 ± 40*	182 ± 40*	588 ± 43†

[a]Mean and S.E.M. x 10^{-2}.

*†Means with the same symbol are not significantly different.

Figure 1. Proventriculus of a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Mucosal and submucosal necrosis, hemorrhage and edema evident. Hematoxylin and eosin. Bar = 1 mm

Figure 2. Ventriculus of a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Mucosal and submucosal necrosis, edema and hemorrhage evident. Hematoxylin and eosin. Bar = 1 mm

Figure 3. Ventriculus of a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Mucosal glands with edema and increased numbers of heterophils. Hematoxylin and eosin. Bar = 40 um

Figure 4. Ventriculus of a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Increased numbers of heterophils associated with swollen mucosal glands and edematous lining. Hematoxylin and eosin. Bar = 40 um

increases in numbers of heterophils and fewer macrophages and eosinophils were also present in the mucosa and submucosa interstitium and around vessels throughout the section (Figure 5). Fibrinous vasculitis was occasionally seen. Also present was acute multifocal epithelial degeneration and erosion of villous tips (Figure 6) limited to the duodenum and jejunum. Villi were also mildly hyperemic with a mild increase in heterophils present in the lamina propria. No remarkable changes were observed in the ileum, ceca and colon. Five of the eight poults in the 5 mg/kg/d group had similar but markedly less pronounced changes in the proventriculus and ventriculus. There were no significant duodenal, jejunal or ileal lesions in the 5 mg/kg/d or control poults.

Hepatic lesions were present in all poults (8/8) in the 10 mg/kg/d group. They consisted of bile duct proliferation (Figure 7) and increased granulocytes and macrophages associated with sinusoids and large bile ducts. A mild multifocal granulomatous hepatitis (Figure 8) was also noted. Slight bile duct proliferation was noted in two (2/8) poults in the 5 mg/kg/d group. No other hepatic changes were noted in either the 5 mg/kg/d or control groups.

No changes were seen in electron photomicrographs of cardiac papillary muscles from any treatment or control poults.

Figure 5. Increased numbers of granulocytes in the submucosa and around a vessel in a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Hematoxylin and eosin. Bar = 40 um

Figure 6. Epithelial degeneration and erosion of duodenal villous tips in a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Hematoxylin and eosin. Bar = 40 um

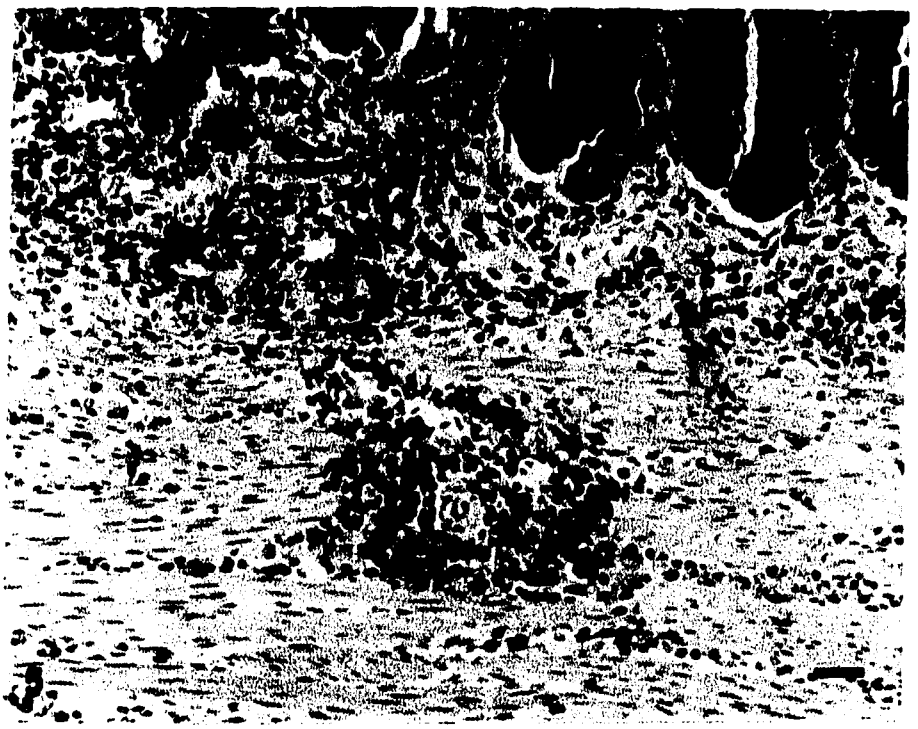
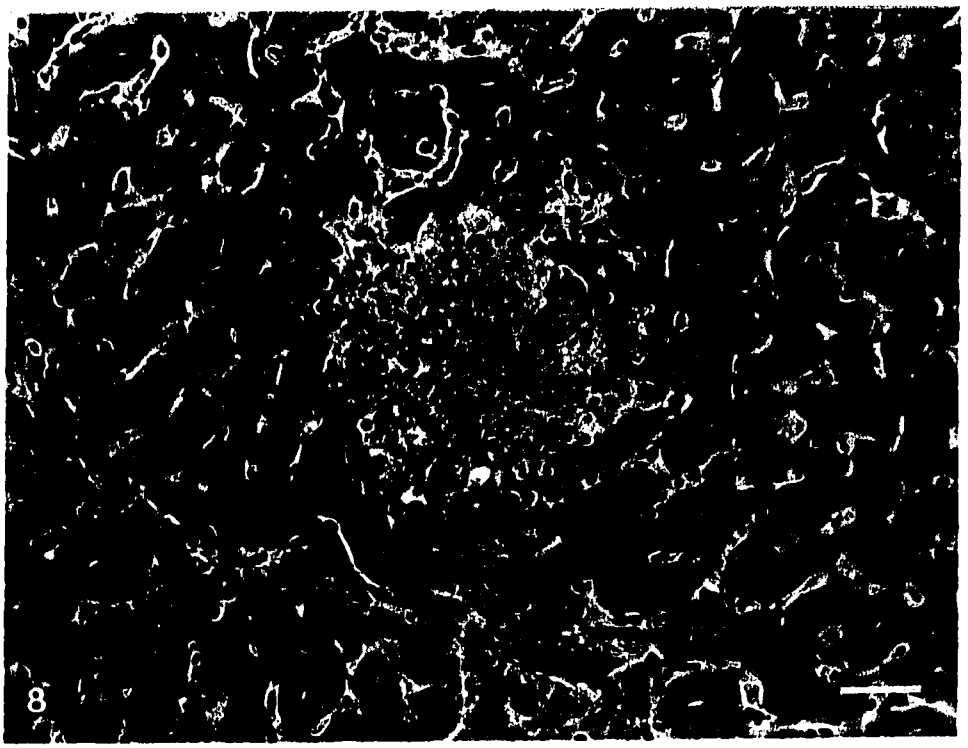


Figure 7. Liver of a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Bile duct proliferation with increased numbers of granulocytes is present. Hematoxylin and eosin. Bar = 40 um

Figure 8. Focal granulomatous hepatitis from a turkey poult given CPA orally at 10 mg/kg/d for 2 days. Hematoxylin and eosin. Bar = 40 um


7


8

DISCUSSION

Acute oral exposure of CPA to turkey poults caused severe clinical signs, hematological changes and histologic lesions. Cyclopiazonic acid caused a significant ($P \leq 0.05$) depression in weight gain, feed consumption and water intake by 24 h, in turkey poults given the 10 mg/kg/d dose. By 48 h, poults in the 5 mg/kg/d group had significantly decreased weight gain and feed consumption. Decreased weight gain and feed intake has also been reported in chickens.[22,25] The decreased water intake, feed intake and weight loss could be related to lesions present in the proventriculus and ventriculus. The edema, thickened lining, and swollen glandular cells was extensive enough to partially prevent deglutition.

Clinical hematologic results were significantly altered at the 10 mg/kg/d exposure level. Both treatment groups had a dose-related leukocytosis and heterophilia with only the 10 mg/kg exposure group increases being statistically significant. A significant lymphocytosis was also present in the highest treatment group. Leukocytosis is often associated with massive tissue necrosis.[33] Gastrointestinal necrosis could have contributed to this response. The leukocytosis with heterophilia and no left shift or increased immature leukocytes suggests that poults in both treatment groups were also stressed. These findings are consistent with the observed gastrointestinal necrosis and lethargy/recumbency. Our findings are consistent with hematological observations of neutrophilia in dogs and rats exposed to CPA,[12,28] and leukocytosis

reported in dogs. However, lymphopenia was also reported in both species following more chronic exposures of 28 days (rat) and 90 days (dogs).

Our histopathologic findings agree with observations in other studies. Purchase reported the effects of oral and intraperitoneal administrations of single doses of CPA in rats included single cell to focal areas of hepatic necrosis. Dorner et al.[22] also reported focal hepatic necrosis and proventricular hyperemia and ulceration in broiler chicks fed CPA contaminated feed for 7 weeks. In another study,[24] oral gavage with CPA at 4 mg/kg for 23 days produced a similar lesion of necrosis and hemorrhage or hyperplasia of proventricular mucosa and hepatocellular vacuolation. The localized necrosis in the upper gastrointestinal tract and liver may indicate a direct toxic effect of CPA. Neuhring and coworkers[28] reported these lesions in a 90 day study with dogs and suggested that this is due to ischemia resulting from vascular damage and thrombosis. Pigs given 10 mg/kg for 14 days had microscopic lesions which included necrotizing gastroenteritis and focal hepatocellular necrosis.[30] Our findings demonstrate that at similar dose levels these lesions occur earlier than has previously been reported.

The absence of myocardial lesions was disappointing. Studies utilizing higher exposure levels have reported cardiac lesions. In an earlier study,[34] in vitro exposure to CPA resulted in mitochondrial ultrastructural changes. Apparently exposing rats[5,7] and monkeys[31] to doses of CPA $\geq$ 12 mg/kg produces cardiac lesions. In our study with

turkeys and studies of rats,[14] chicks,[24] dogs[28] and piglets[30] exposed to CPA levels $\leq$ 10 mg/kg, no cardiac lesions were noted.

The 3 *in vivo* studies in which there was reported cardiac lesions were of longer duration, with total oral doses exceeding that of the present study's dose of 10 mg CPA/kg. Perhaps a longer duration of dosing using doses similar to those utilized in this study or higher doses could result in cardiac lesions in turkeys. However, the doses utilized in this study resulted in a severe clinical response and graphic histopathologic changes in the gastrointestinal tract and liver which would preclude doses this high in this species.

REFERENCES

1. Gallagher RT, Richard JL, Stahr MH, et al. Cyclopiazonic acid production by aflatoxigenic and non-aflatoxigenic strains of Aspergillus flavus. Mycopathologia 66:31-36, 1978.

2. Lansden J, Davidson J. Occurrence of cyclopiazonic acid in peanuts. Appl Environ Microbiol 45:766-769, 1983.

3. Still P, Eckardt C, Leistner L. Bildung von cyclopiazonsaure durch Penicillium camemberti-isolate von kase. Fleischwirtschaft 58: 876-877, 1978.

4. Rao BL, Husain A. Presence of cyclopiazonic acid in Kodo Millet (Paspalum scrobiculatum) causing "Kodua poisoning" in man and its production by associated fungi. Mycopathologia 89:177-180, 1985.

5. Purchase IFH. The acute toxicity of the mycotoxin cyclopiazonic acid to rats. Toxicol Appl Pharmacol 18:114-123, 1971.

6. Norred WP, Morrissey RE, Cole RJ, et al. Hepatotoxic effects of cyclopiazonic acid in rats. Fed Proc 43:578, 1984.

7. Van Rensburg SJ. Subacute toxicity of the mycotoxin cyclopiazonic acid. Fd Chem Toxicol 22:993-998, 1984.

8. Morrissey RE, Cole RJ, Dorner JW. The effects of cyclopiazonic acid on pregnancy and fetal development of fischer rats. J Toxicol Environ Health 14:585-594, 1984.

9. Hinton DM, Morrissey RE, Norred RE, et al. Effects of cyclopiazonic acid on the ultrastructure of rat liver. Toxicol Lett 25:211-218, 1985.

10. Morrissey RE, Norred WP, Cole RJ, et al. Toxicity of the mycotoxin, cyclopiazonic acid, to sprague-dawley rats. Toxicol Appl Pharmacol 77:94-107, 1985.

11. Norred WP, Morrissey RE, Riley RT. Distribution, excretion and skeletal muscle effects of the mycotoxin [^{14}C] cyclopiazonic acid in rats. Food Chem Toxicol 23:1069-1076, 1985.

12. Hill JE, Lomax LG, Cole RJ, et al. Toxicologic and immunologic effects of sublethal doses of cyclopiazonic acid in rats. Am J Vet Res 47:1174-1177, 1986.

13. Rathinavely A, Shanmugasundaram ER, Shanmugasundaram ERB. Studies on rat liver hexokinanse during cyclopiazonic acid toxicosis. Indian J Exp Biol 24:179-181, 1986.

14. Morrissey RE, Norred WP, Hinton DM, et al. Combined effects of the mycotoxins aflatoxin B_1 and cyclopiazonic acid on sprague-dawley rats. Food Chem Toxicol 25:837-842, 1987.

15. Nishi K, Porter JK, Cole RJ, et al. Neurochemical and pharmacological effects of cyclopiazonic acid, chlorpromazine and reserpine. Res Commun Chem Pathol Pharmacol 10:291-302, 1985.

16. Nishie K, Porter JK, Cole RJ, et al. Toxicity and neuropharmacology of cyclopiazonic acid. Food Chem Toxicol 23:831-839, 1985a.

17. Khera KS, Cole RJ, Whalen C, et al. Embryo-toxicity study on cyclopiazonic acid in mice. Bull Environ Contam Toxicol 34:423-426, 1985.

18. Nishie K, Cole RJ, Dorner JW. Toxic effects of cyclopiazonic acid in early phase of pregnancy in mice. Res Commun Chem Pathol Pharmacol 53:303-315, 1987.

19. Peden WM. Effect of cyclopiazonic acid on guinea pig skeletal muscle. Abstr Pap Symp Cellular and Molecular Mode of Action of Selected Microbial Toxins in Foods and Feed, Chevy Chase, MD, 1988.

20. Pier AC, Belden EL, Ellis JH, et al. Interaction between aflatoxin and cyclopiazonic acid on clinical pathological and immunological responses of guinea pigs. Abstr Pap Annu Meet Conf Res Work Anim Dis 68: 157, 1987.

21. Richard JL, Peden WM, Fichtner RF, et al. Effect of cyclopiazonic acid on delayed hypersensitivity to Mycobacterium tuberculosis, complement activity, serum enzymes, and bilirubin in guinea pigs. Mycopathologia 96:73-77, 1986.

22. Dorner JW, Cole RJ, Lomax LG, et al. Cyclopiazonic acid production by Aspergillis flavus and its effects on broiler chickens. Appl Environ Microbiol 46:698-703, 1983.

23. Vesley D, Vesela D, Neumannova V. Cyclopiazonic acid production: its effects on chicken embryos and day-old cockerels. Vet Med 30:345-352, 1985.

24. Cullen JM, Wilson M, Hagler WM, et al. Histologic lesions in broiler chicks given cyclopiazonic acid orally. Am J Vet Res 49:728-731, 1988.

25. Norred WP, Porter JK, Dorner JW, et al. Occurrence of the mycotoxin cyclopiazonic acid in meat after oral administration to chickens. J Agric Food Chem 36:113-116, 1988.

26. Porter JK, Norred WP, Cole RJ, et al. Neurochemical effects of cyclopiazonic acid in chickens. Proc Soc Exp Biol Med 187:335-340, 1988.

27. Wilson ME, Hagler WM, Ort JF, et al. Acute and subacute effects of cyclopiazonic acid in broiler chickens. Poultry Sci 65:145, 1986.

28. Neuhring LP, Rowland GN, Harrison LR, et al. Cyclopiazonic acid mycotoxicosis in the dog. Am J Vet Res 46:1670-1676, 1985.

29. Nishie K, Cole RJ, Dorner JW. Neurological and cardiac effects of cyclopiazonic acid. Abstr Pharmacol 26:536, 1984.

30. Lomax LG, Cole RJ, Dorner JW. The toxicity of cyclopiazonic acid in weaned pigs. Vet Pathol 21:418-424, 1984.

31. Jaskiewicz K, Close PM, Thiel PC, et al. Preliminary studies on toxic effects of cyclopiazonic acid alone and in combination with aflatoxin B_1 in non-human primates. Toxicology 52:297-307, 1988.

32. Miller CD, Richard JL, Osweiler GD, et al. The toxicity of cyclopiazonic acid in turkey poults; clinical, physiological and serological observations. Manuscript in preparation. National Animal Disease Center, Ames, IA.

33. Campbell TW, ed. In: Avian Hematology and Cytology. First ed. Ames:Iowa State University Press, 1988; 17.

34. Miller CD, Osweiler GD, Richard JL, et al. Ultrastructural and functional changes of turkey myocardium induced by *in vitro* exposure to cyclopiazonic acid. Manuscript in preparation. National Animal Disease Center, Ames, IA.

GENERAL SUMMARY AND DISCUSSION

The toxicity of CPA has been demonstrated in several animal species including rats, mice, guinea pigs, dogs, rabbits, pigs and monkeys.[1-21,24-27] There are a few studies of CPA toxicity in chickens;[18-20,22,23] however, no information is available regarding the toxicity of CPA in turkeys. In this study, turkey heart muscle was utilized as a model to test the acute *in vitro* effects of CPA on isolated muscle performance. Muscle performance was evaluated and correlated with pathologic changes. The *in vivo* effects of acute CPA exposure were studied in turkey poults.

In the first study, it was demonstrated that an *in vitro* biological assay system, that utilized turkey papillary muscle, could function in an isolated chamber containing oxygenated, pH-regulated, modified Krebs-Henseleit solution. Baseline information on *in vitro* performance of turkey papillary muscle preparations was established. It was then demonstrated that different calcium concentrations affect muscle contraction and relaxation velocities, load values and latencies.

In the second study, the *in vitro* biological assay system was utilized to evaluate the effects of cyclopiazonic acid mycotoxin on cardiac muscle performance. Acute exposure to CPA at 6 ug/ml of bathing solution significantly decreased five performance criteria of *in vitro* papillary muscle. These criteria were: maximum weight a muscle could lift, maximum contraction velocity, maximum relaxation velocity, time to peak contraction and total time for muscle contraction and

relaxation. These altered performances appeared to be intracellular changes partially associated with calcium availability and were irreversible suggesting that physiopathological changes occurred following acute CPA exposure.

Results of the third study further characterized the observed decrease in muscle performance seen in the second study and correlated *in vitro* muscle performance with pathological changes. Isolated papillary muscles mounted in the biological assay chamber were exposed acutely to 6 ug CPA/ml of bathing solution. Seven parameters of *in vitro* papillary muscle performance were significantly decreased. The seven performance criteria were; maximum weight a muscle could lift, work, maximum contraction velocity, maximum relaxation velocity and times to initiation, peak and completion of contractions. Electron photomicrographs demonstrated that the CPA exposed muscles, evaluated in the biological assay system, had increased numbers of swollen or lysed mitochondria. Irreversible decreased muscle performance and altered mitochondrial morphology were attributed to altered calcium and ATP availability.

The fourth study was designed to establish a dose response for turkey poults and represents the first report of CPA toxicity in turkey poults. Clinical signs were similar to those of chickens and included lethargy, ataxia, drooped head and wings, ruffled feathers, marked anorexia, and watery feces of reduced amount. Five turkeys given 20 mg CPA/kg/d were killed *in extremis*. Total serum proteins and albumin were decreased by CPA ingestion. Cyclopiazonic acid was found in feces

collected during the first 24 h period and corresponded to dosage levels. The major gross lesions noted at necropsy occurred in the gastrointestinal tract and liver. Poults receiving 10 and 20 mg/kg/d had focal erosions in the proventriculus and ventriculus, a fibrinogelatinous membrane present in the ventriculus, and increased liver weights. The clinical and pathological findings from this study would support the retrospective conjecture of Cole[33] that CPA could have been involved in "Turkey X disease."

Based on the results from the third and fourth study, we then tested the hypothesis that ingestion of either 5 or 10 mg CPA/kg/d by turkey poults for 2 days would produce cardiac myocyte ultrastructural changes similar to those seen in the in vitro study. We also evaluated pathological and hematological changes. The results of the fifth study proved this hypothesis was incorrect.

In the fifth study, changes caused by CPA included leukocytosis, heterophilia, and lymphocytosis, and histopathologic changes of mucofibrinous necrotizing proventriculitis and ventriculitis, acute multifocal epithelial degeneration and erosion of villous tips and mild multifocal granulomatous hepatitis with bile duct proliferation. Body weight, feed consumption and water intake were significantly decreased by CPA ingestion. No cardiac ultrastructural changes were noted.

These studies point out that in vitro experiments provide information that is difficult to extrapolate to in vivo settings. Questions arise. What are the differences between in vitro and in vivo metabolism of CPA?, Do muscles bathed in chamber solution present different

binding sites for CPA?, What *in vivo* exposure level corresponds to 6 ug CPA/ml bathing solution?

These studies have contributed to a better understanding of pathologic, hematologic, and serologic changes in turkey poults given oral CPA mycotoxin.

LITERATURE CITED

1. Purchase IFH. The acute toxicity of the mycotoxin cyclopiazonic acid to rats. Toxicol Appl Pharmacol 18:114-123, 1971.

2. Norred WP, Morrissey RE, Cole RJ, et al. Hepatotoxic effects of cyclopiazonic acid in rats. Fed Proc 43:578, 1984.

3. Van Rensburg SJ. Subacute toxicity of the mycotoxin cyclopiazonic acid. Fd Chem Toxicol 22:993-998, 1984.

4. Morrissey RE, Cole RJ, Dorner JW. The effects of cyclopiazonic acid on pregnancy and fetal development of fischer rats. J Toxicol Environ Health 14:585-594, 1984.

5. Hinton DM, Morrissey RE, Norred RE, et al. Effects of cyclopiazonic acid on the ultrastructure of rat liver. Toxicol Lett 25:211-218, 1985.

6. Morrissey RE, Norred WP, Cole RJ, et al. Toxicity of the mycotoxin, cyclopiazonic acid, to sprague-dawley rats. Toxicol Appl Pharmacol 77:94-107, 1985.

7. Norred WP, Morrissey RE, Riley RT. Distribution, excretion and skeletal muscle effects of the mycotoxin [^{14}C] cyclopiazonic acid in rats. Food Chem Toxicol 23:1069-1076, 1985.

8. Hill JE, Lomax LG, Cole RJ, et al. Toxicologic and immunologic effects of sublethal doses of cyclopiazonic acid in rats. Am J Vet Res 47:1174-1177, 1986.

9. Rathinavely A, Shanmugasundaram ER, Shanmugasundaram ERB. Studies on rat liver hexokinanse during cyclopiazonic acid toxicosis. Indian J Exp Biol 24:179-181, 1986.

10. Morrissey RE, Norred WP, Hinton DM, et al. Combined effects of the mycotoxins aflatoxin B_1 and cyclopiazonic acid on sprague-dawley rats. Food Chem Toxicol 25:837-842, 1987.

11. Nishi K, Porter JK, Cole RJ, et al. Neurochemical and pharmacological effects of cyclopiazonic acid, chlorpromazine and reserpine. Res Commun Chem Pathol Pharmacol 10:291-302, 1985.

12. Nishie K, Porter JK, Cole RJ, et al. Toxicity and neuropharmacology of cyclopiazonic acid. Food Chem Toxicol 23:831-839, 1985a.

13. Khera KS, Cole RJ, Whalen C, et al. Embryo-toxicity study on cyclopiazonic acid in mice. Bull Environ Contam Toxicol 34:423-426, 1985.

14. Nishie K, Cole RJ, Dorner JW. Toxic effects of cyclopiazonic acid in early phase of pregnancy in mice. Res Commun Chem Pathol Pharmacol 53:303-315, 1987.

15. Peden WM. Effect of cyclopiazonic acid on guinea pig skeletal muscle. Abstr Pap Symp Cellular and Molecular Mode of Action of Selected Microbial Toxins in Foods and Feed, Chevy Chase, MD, 1988.

16. Pier AC, Belden EL, Ellis JH, et al. Interaction between aflatoxin and cyclopiazonic acid on clinical pathological and immunological responses of guinea pigs. Abstr Pap Annu Meet Conf Res Work Anim Dis 68: 157, 1987.

17. Richard JL, Peden WM, Fichtner RF, et al. Effect of cyclopiazonic acid on delayed hypersensitivity to Mycobacterium tuberculosis, complement activity, serum enzymes, and bilirubin in guinea pigs. Mycopathologia 96:73-77, 1986.

18. Dorner JW, Cole RJ, Lomax LG, et al. Cyclopiazonic acid production by Aspergillis flavus and its effects on broiler chickens. Appl Environ Microbiol 46:698-703, 1983.

19. Vesley D, Vesela D, Neumannova V. Cyclopiazonic acid production: its effects on chicken embryos and day-old cockerels. Vet Med 30:345-352, 1985.

20. Cullen JM, Wilson M, Hagler WM, et al. Histologic lesions in broiler chicks given cyclopiazonic acid orally. Am J Vet Res 49:728-731, 1988.

21. Norred WP, Porter JK, Dorner JW, et al. Occurrence of the mycotoxin cyclopiazonic acid in meat after oral administration to chickens. J Agric Food Chem 36:113-116, 1988.

22. Porter JK, Norred WP, Cole RJ, et al. Neurochemical effects of cyclopiazonic acid in chickens. Proc Soc Exp Biol Med 187:335-340, 1988.

23. Wilson ME, Hagler WM, Ort JF, et al. Acute and subacute effects of cyclopiazonic acid in broiler chickens. Poultry Sci 65:145, 1986.

24. Neuhring LP, Rowland GN, Harrison LR, et al. Cyclopiazonic acid mycotoxicosis in the dog. Am J Vet Res 46:1670-1676, 1985.

25. Nishie K, Cole RJ, Dorner JW. Neurological and cardiac effects of cyclopiazonic acid. Abstr Pharmacol 26:536, 1984.

26. Lomax LG, Cole RJ, Dorner JW. The toxicity of cyclopiazonic acid in weaned pigs. Vet Pathol 21:418-424, 1984.

27. Jaskiewicz K, Close PM, Thiel PC, et al. Preliminary studies on toxic effects of cyclopiazonic acid alone and in combination with aflatoxin B_1 in non-human primates. Toxicology 52:297-307, 1988.

28. Gallagher RT, Richard JL, Stahr MH, et al. Cyclopiazonic acid production by aflatoxigenic and non-aflatoxigenic strains of Aspergillus flavus. Mycopathologia 66:31-36, 1978.

29. Lansden J, Davidson J. Occurrence of cyclopiazonic acid in peanuts. Appl Environ Microbiol 45:766-769, 1983.

30. Still P, Eckardt C, Leistner L. Bildung von cyclopiazonsaure durch Penicillium camemberti-isolate von kase. Fleischwirtschaft 58: 876-877, 1978.

31. Le Bars J. Cyclopiazonic acid production by Penicillium camemberti Thom and natural occurrence of this mycotoxin in cheese. Appl Environ Microbiol 38:1052-1055, 1979.

32. Holzapfel CW. The isolation and structure of cyclopiazonic acid, a toxic metabolite of Penicillium cyclopium Westling. Tetrahedron 24:2109-2119, 1968.

33. Cole RJ. Etiology of turkey "x" disease in retrospect: a case for the involvement of cyclopiazonic acid. Mycotoxin Res 2:3-7, 1986.

34. Holzapfel CW, Wilkins DC. On the biosynthesis of cyclopiazonic acid. Phytochemistry 10:351-358, 1971.

35. Cole RJ, Cox RH. In: Handbook of Toxic Fungal Metabolites. New York:Academic Press, 497-509, 1981.

36. Holzapfel CW, Hutchison RD, Wilkins DC. The isolation and structure of two new indole derivatives from Penicillium cyclopium Westing. Tetrahedron 26:5239-5246, 1970.

37. McGrath RM, Steyn PS, Ferreira NP, et al. Biosynthesis of cyclopiazonic acids in Penicillium cyclopium: the isolation of dimethylallylpyrophosphate: cyclo-acetoacetyltryptophanyl dimethylallytransferase. Bioorg Chem 5:11-23, 1976.

38. LeBars J. Cyclopiazonic acid bioproduction by Penicillium camemberti Thom effect of temperature on individual strains. Ann Rech Vet 10:601-602, 1979.

39. Leistner L, Pitt JI. Miscellaneous Penicillium toxins. In: Mycotoxins in Human and Animal Health. Park Forest South, IL: Pathotox Publishers Inc, 639-653, 1977.

40. Trucksess MW, Mislivec PB, Young K, et al. Cyclopiazonic acid production by cultures of Aspergillus and Penicillium species isolated from dried beans, corn meal, macaroni, and pecans. J Assoc Off Anal Chem 70:123-126, 1987.

41. Lansden JA. Determination of cyclopiazonic acid in peanuts and corn by thin layer chromatography. J Assoc Off Anal Chem 69: 964-966, 1986.

42. Benkhemmar O, Gaudemer F, Bouvier-Fourcade I. Heterokaryosis between Aspergillus oryzae cyclopiazonic acid-defective strains: method for estimating the risk of inducing toxin production among cyclopiazonic acid-defective industrial strains. Appl Environ Microbiol 50:1087-1093, 1985.

43. Goto T, Shinshi E, Tanakak, et al. Analysis of cyclopiazonic acid by normal phase high-performance liquid chromatography. Agric Biol Chem 51:2581-2582, 1987.

44. Rao BL, Husain A. A simple colorimetric method for screening cyclopiazonic acid in agricultural commodities. Mycopathologia 97: 89-92, 1987.

45. Wehner FC, Thiel PG, Van Rensbert SJ, et al. Mutagenicity to Salmonella typhimurium of some Aspergillus and Penicillium mycotoxins. Mutat Res 58:193-203, 1978.

46. Sorenson WG, Tucker JD, Simpson JP. Mutagenicity of the tetramic mycotoxin cyclopiazonic acid. Appl Environ Microbiol 47:1355-1357, 1984.

47. Isaacson C. The aetiology of chirrhosis and hepatoma in the Bantu--an appraisal. S Afr Med J 40:11-13, 1966.

48. Nishie K, Cole RJ, Dorner JW. Effects of cyclopiazonic acid on the contractility of organs with smooth muscles, and on frog ventricles. Res Commun Chem Pathol and Pharmacol 53:23-37, 1986.

49. Riley RT, Showker JL, Cole RJ, et al. The mechanism by which cyclopiazonic acid potentiates accumulation of tetraphenylphosphonium in cultured renal epithelial cells. J Biochem Toxicol 1:13-29, 1986.

50. Riley RT, Norred WP, Dorner JW, et al. Increased accumulation of the lipophilic cation tetraphenylphosphonium^{+} by cyclopiazonic acid-treated renal epithelial cells. J Toxicol Environ Health 15:779-788, 1985.

51. Riley RT, Goeger DE, Norred WP, et al. Age and growth-related changes in cyclopiazonic acid-potentiated lipophilic cation accumulation by cultured cells and binding to freeze-thaw lysed cells. J Biochem Toxicol 2:251-264, 1987.

52. Goeger DE, Riley RT, Dorner JW, et al. Cyclopiazonic acid inhibition of the CA^{2+}-transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles. Biochem Pharmacol 37:978-981, 1988.

53. Richard JL, Peden WM, Thurston JR. In: Cellular and Molecular Mode of Action of Selected Microbial Toxins. New York: Plenum Press, Accepted Nov. 1988.

ACKNOWLEDGMENTS

I wish I could cite and thank the many individuals who contributed to the completion of this degree. However, I will limit my remarks to those that contributed, supported and encouraged me the most during this experience.

No words can adequately express my gratitude to my wife Rita for her encouragement, patience and understanding. A special thanks to my children, Ryan and Amber who have always forgiven me for the times I was not there.

To my co-major professors, Dr. Gary D. Osweiler and Dr. John L. Richard, I thank you for allowing me to continue my pursuit of an advanced degree. You both contributed in augmenting ways to assist my research and writing efforts. I have valued your instruction, encouragement and constructive criticism. I gained from your expressed philosophies the insight of what it takes to become a scientist. I value this rich heritage.

I have been further enriched by participating in the Toxicology Interdepartmental Program, initially chaired by Dr. Joel R. Coats and currently under the guidance of Dr. Gary D. Osweiler. Further thanks are expressed to Drs. David F. Cox, Thomas L. Carson and Lyle D. Miller for serving as excellent committee members. Your assistance and advice throughout my educational experience is greatly appreciated.

Sincere gratitude is expressed to the former Director of the National Animal Disease Center, Dr. Phillip A. O'Berry, my former

supervisor, Joseph R. Songer, my current supervisor, Dr. Wayne M. Frerichs and the Director of the National Veterinary Services Laboratories, Dr. Robert M. Nervig, for their support, vision and facilities which made this research possible.

Appreciation is also expressed to Dr. W. Michael Peden for necropsy assistance and general consultation, Dr. Vincent P. Meador for pathologic consultations and Dr. Frederick B. Hembrough for use of laboratory space and constructive manuscript review.

A special thanks is expressed for the technical assistance provided by: Barbara J. Royer, Lonna J. Nielsen, Rebecca L. Lyon, Rodney E. Fichtner, and Nudith A. Stasko.

To the NADC librarians, animal supply personnel and photographic unit personnel I am indebted. I also wish to express my appreciation to Karen A. Burianek for typing this dissertation and her constant encouragement.